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DEVELOPMENT OF LIVER CANCERS IN THE RAT BY 20-METHYLCHOLANTHRENE PAINTING FOLLOWING INITIAL 4-DIMETHYLAMINOAZOBENZENE FEEDING

(Plates XIX～XXIV)

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INTRODUCTION

Recently many reports have been made on the experiments which supported the concept of two-phase mechanism of carcinogenesis first advocated by I. Berenblum[1] and also by P. Rous and J. D. Kidd.[2] According to the former, skin tumors in mice were formed by the combined application of 3, 4-benzpyrene and croton oil, while the latter authors found that skin tumors in a rabbit's ear developed through the combined application of coal tar and mechanical irritants. These results were interpreted as showing that, after the treatment of the skin with subcarcinogenic doses of a carcinogenic hydrocarbon, which are insufficient to produce cancer, various kinds of non-carcinogenic irritants could develop skin tumors, when these were given to the pretreated site of skin. In this concept, the former process is called "Initiation", while the latter "Promotion".

Subsequently, Graffi et al.[3] and Salaman and Roe.[4] found that the urethan painting on the skin of mice, previous to, or alternate with, the croton treatment, caused many benign papillomata, and some of those happened to change into carcinomas. They found that the application of urethan by routes other than the percutaneous could bring about the same effect. Moreover, much progress has been made in which new initiating agents of non-carcinogenic hydrocarbon are applied.[5),6)]

On the other hand, in regard to the histological differences between the tumors induced by the combined method and the tumors caused only by a single carcinogen, Shubik,[7] Salaman and Roe,[8] and some others reported that most of the tumors produced were benign by the former method while skin carcinomas were formed in most cases by the latter method. When the promoting treatment was stopped, some of those benign tumors showed spontaneous regression, some maintained the status, and some continued the growth showing malignant conversion.

Bielschowsky[9] and Hall[10] made similar experiments independenly with neoplasms other than skin tumors. In their cases, the application of 2-acethylaminofluorene and allylthiourea or 4-methyl 2-thiouracil could cause the formation of thyroid

tumors. Meanwhile, Glinos[11] et al. observed that liver cancers were induced after partial hepatectomy following a short 4-dimethylaminoazobenzene (DAB) treatment. In this case, they regarded DAB as the initiating agent, and the post-operative regeneration of the liver as the promoting factor. From the experiments of intramuscular injection with 20-methylcholanthrene dissolved in olive oil and with croton oil in olive oil, Klein[12] reported that two-phase mechanism was suggested in the development of subcutaneous tumors.

In Japan, very few reports have been made concerning this conception, except Muta's experiments.[13),14)] In his experiments the application of o-aminoazotoluene (AAT) was combined with coal tar, with 3, 4-benzpyrene, and with arsenic trioxide. He reported that, the painting of coal tar on the skin caused the formation of liver cancers and 2 examples of gastric carcinomas after the oral application of AAT to albino rats for 3 months.

From the above experiments, it is affirmed that a non-neoplastic change initiated in a tissue by application of subcarcinogenic doses of some kind of carcinogenic agents, could develop and cause the fomation of tumors by means of the action of other kinds of irritants——the irritants unable to cause, by themselves, cancerous changes in the tissues or organs.

In this present experiment, the administration of 4-dimethylaminoazobenzene was followed by 20-methylcholanthrene (MC) painting. The histological changes of the liver were studied at various combinations of the DAB-feedings and MC-paintings. The reversible and irreversible changes induced in the liver by DAB-feeding were investigated, especially the initial changes for the development of liver cancers were examined without further feeding of DAB. Furthermore, the role of the percutaneous application of MC was investigated for cancer development in the liver of the animals which were given insufficient dose of DAB for induction of liver cancers. Based on the results obtained, this report deals with a fundamental discussion about the concept of two-phase mechanism of carcinogenesis.

In addition, the histo-pathological and biological characteristics of the induced liver cancers will be described, especially based on metastatic ability and transplantability of the tumors.

Materials and Methods

Animals: The animals used in the present experiments were Japanese common albino rats, including 151 females and 250 males. The animals of each sex were housed in wooden animal cages, 2 to a cage, and their initiating body weight was from 80 to 120 g.

Basic diet: A semi-synthetic cube diet (Oriental Co.) was used.

DAB-diet: The powdered basic diet containing DAB (m. p. 117°C) was made into tablets weighing 1.9 g each. The animals took the tablets ad libitum and each rat was fed with ca. 10–15 g of the diet a day. The DAB-diet was composed of 0.6 g of 4-dimethylaminoazobenzene, 550 g of carbohydrate, 186 g of protein, 44 g of fat, 74 g of salt mixture, 52 g of water, 74 g of gum arabic, and 20 g of precipitated calcium carbonate, per kg. DAB was dissolved in ethyl-alcohol and mixed with the basic diet. Gum arabic and precipitated calcium carbonate were added as the excipients.

20-Methylcholanthrene painting: 0.3% 20-methylcholanthrene (Kodak Co., Ltd.) solution in acetone was painted, twice a week, on the shaved interscapular region of the animals.

Experimental groups: The albino rats were divided into 5 groups (Group I–V), and each group received the following treatments.

Group I (56 rats): Immediately after one month of DAB-feeding, 11 of them (subgroup A) were sacrificed, and their histo-pathological liver canges were examined. The 45 survivors were fed with the basic diet. Three days after the stop of the DAB-feeding, these survivors were divided into subgroups B (18) and C (27). The animals in subgroup C received MC-painting for 8 months in the way stated above.

Group II (55 rats): After 2 months of DAB-feeding, 10 (subgroup A) were sacrificed for histological examination of the liver. After 3 days, the 37 surviving rats were divided into subgroups B (13) and C (24), and fed with the basic diet. The animals in subgroup C received MC-painting for 7 months.

Group III (75 rats): After 3 months of DAB-feeding, 16 were sacrificed for autopsy (subgroup A). After 3 days, the 48 survivors were divided into subgroups B (17) and C (31). The animals of the latter group received MC-painting for 6 months.

Group IV (75 rats): After 4 months of DAB-feeding, 12 were sacrificed for microscopical examination (subgroup A). After 3 days, the 50 survivors were divided into subgroups B (19) and C (31). The animals in subgroup C received MC-painting for 5 months.

Group V (100 rats): After 5 month of DAB-feeding, 17 of them were sacrificed for autopsy (subgroup A). After 3 days, the 62 surviving rats were divided into subgroups B (30) and C (32). The animals in subgroup C received MC-painting for 4 months.

The general schedules of the experiment were summarized in Table 1.

Experimental procedure: Throughout the whole groups, the first liver cancer was detected after 210 experimental days. Then all the survivors were examined, by palpation, for the presence of liver tumors once in every week. The animals

Table 1. Term of the treatment and number of the animals.

Group	Months of		Number of animals			
	DAB-feeding	MC-painting	Total	Early death		Valids
				during DAB-feeding	before 210 expl. days	
I-A	1	—	56	0	—	11
B		0			8	10
C		8			7	20
II-A	2	—	55	8	—	10
B		0			2	11
C		7			6	18
III-A	3	—	75	11	—	16
B		0			4	13
C		6			11	20
IV-A	4	—	75	13	—	12
B		0			4	15
C		5			5	26
V-A	5	—	100	21	—	17
B		0			4	26
C		4			3	29

A: Sacrificed immediately after the stop of the DAB-feeding.
B: Fed with basic diet following the DAB-feeding.
C: MC-painting following the DAB-feeding.
The same remarks used in the following tables.

in which tumors were palpated in their right hypochondrial region were put under observation for 1 to 3 weeks untill the tumors were enlarged and the animals were debilitated. When the rats fell into agonized condition, they were sacrificed under ether narcose, and examined for the presence of the liver cancers or the spontaneous tumor ascites. When tumors were noticed, the intraperitoneal transplantation was carried out either with the minced tumor nodules or with the tumor ascites to investigate the transplantability of the induced tumors. Among the sacrificed animals regarded as tumor positive by palpation, there were 4 in which the formation of liver cancers could not be found. In 2 of these 4, the abscess formation in the right lung was observed in a high degree, and in the other 2, sarcomas of the liver which might have developed spontaneously around the cysticercus cysts, was revealed.

Four hundred and twenty days after the start of the experiment, all the survivors were sacrificed and subjected for autopsy.

Histo-pathological examinations: The liver, kidneys, spleen, pancreas, lungs, mesenterial lymph nodes, thymus, mediastinal lymph nodes, and the metastatic tumor nodules were examined. These were fixed with Orth's solution and were made into paraffin specimens. The staining employed were hematoxylin-eosin staining,

Azan-staining and PAS-staining after glycogen digestion, for the purpose of microscopical examinations.

Results

A) HISTO-PATHOLOGICAL FINDINGS OF THE LIVER IMMEDIATELY AFTER THE STOP OF DAB-FEEDINGS OF VARIOUS PERIODS.

The average liver weight and index of the animals in each group sacrificed immediately after the stop of DAB-feeding are tabulated in Table 2. It was observed that there was a tendency that the longer the period of DAB-feelding was, the more their average index increased.

Table 2. Average liver weight and index of the rats which were killed immediately after the stop of the DAB-feeding.

Group	Animals	Liver weight (min.-max.)	Liver index (min.-max.)
I-A	11	8.9 gr. (7.2-9.8)	5.3 (4.3-6.6)
II-A	10	8.9 gr. (5.4-9.9)	5.3 (4.4-6.4)
III-A	16	10.1 gr. (7.3-12.9)	5.7 (5.1-6.9)
IV-A	12	10.1 gr. (6.3-12.5)	6.2 (5.1-8.6)
V-A	17	14.7 gr. (6.8-15.5)	6.2 (5.4-8.6)

I) One month of DAB-feeding (Group I-A): In all the cases, a remarkable proliferation of bile ducts around Glisson's capsule was noticed, and it was found mainly in the interlobular zone. In 2 cases, proliferation was found also intralobularly. But the acinus itself generally kept a normal structure. By means of PAS-staining, pinkish homogenous substances were observed in the lumen of those proliferated bile ducts. In most liver cells, enlargement of cells and nuclei, increase of mitotic figures, pycnosis of nuclei, and a slight fatty degeneration were observed. Central veins were found to be dilated.

II) Two months of DAB-feeding (Group II-A): The bile ducts showed a remarkable proliferation extending intraacinarily and intercellularly, and it was not rare to see the case where one hepatic cell or its clusters were surrounded by them. On the other hand, the proliferated bile ducts in and around the Glisson's capsules were found to compose adenomatous structures and it was also found, in 4 cases out of 10, that those bile ducts, accompanied with proliferated connective tissue cells, showed an early stage of what is called cholangiofibrosis (Fig. 1). However, by means of Azan-staining, increase of collagenous fibers could not be noticed yet in the field. By PAS-staining, those bile ducts which formed the field of cholangiofibrosis were demonstrated to be full of deep violet-stained substances. The substances were often found either in the cavities of the increased small bile ducts at the acinus, or in the cytoplasms of the epithelia, of which those small bile ducts were composed.

It was found that, on account of the proliferation of bile ducts, the liver cell cords lost their normal structure and were divided into large and small groups of cells, and that these groups of irregular liver cell cords surrounded by the bile ducts and the intraacinary liver cells under a normal structure, generally showed degenerative figures slightly, such as disappearance of glycogen, formation of vacuolus, a slight staining of cytoplasms, pycnosis of nuclei, and irregularity of nuclei and cell bodies in size (Fig. 2). On the other hand, a number of small foci of proliferated hepatic cells showing many mitotic figures were found to be dispersed in haphazared regions. The hepatic cells which composed the proliferating field were, usually, smaller in size and larger in nuclear ratio than normal liver cells, and their nuclei were abundant in chromatin. The cytoplasms were stained clear and acidophilic.

III) Three months of DAB-feeding (Group III-A): Increase of bile ducts was shown in a higher degree, and acini under normal structure could hardly be found. Formation of cholangiofibrosis was accelerated, and, in some cases, the lesions were observed in the whole lobes of the liver. In these cases, there were 3 in which small clusters of enlarged liver cells could be found sporadically among the adenomatously proliferated bile ducts. In one of these 3 cases, it was found that the stainability of the cells composing the ducts was intensified; and that, those cells papillomatously proliferated inside the ducts, or increased in the surroundidg stromas and resulted in the disorder of their basement membrane. PAS-staining showed that those ducts were full of deep violet substances. Further, 3 cases of cyst formation were found, in which the cysts were surrounded by a flat or cuboidal layer of bile duct cells (Fig. 3).

On the other hand, the changes of hepatic cells were essentially similar to those which were observed in the rats of Group II-A, but the degree of the proliferating changes were remarkably advanced. Comparatively small hepatic cells, which were abundant in chromatin and possessed distinct nuclei, were found everywhere among the transformed acini, and in some parts, the presence of sinusoid could hardly be noticed because of those compact hepatic cell cords. In 13 cases, such figures as what is called nodulary hyperplasia could be noticed. Even in such parts, however, liver cell cords formed only one layer, and their cytoplasms were stained rather eosinophilic.

IV) Four months of DAB-feeding (Group IV-A): A characteristic liver change in the animals of this group was the appearance of small nodules composed of the cells which had relatively small cell bodies, comparatively enlarged nuclei and slightly basophilic cytoplasms, together with the proliferating liver cells as observed in the liver of Group III-A. They proliferated expansively, pressing and sometimes entering among the hepatic cell cords around them (Figs. 4 and 5). Macro-

scopic examination showed that some of those nodules of what is called adenomatous hyperplasia exceeded the miliary size and could not be distinguished from small adenoma or hepatoma nodules. These findings were obtained in 7 cases. In some cases, nodular hyperlasia increased in number and size.

On the other hand, such degenerating figures of hepatic cells as observed in the rats of Group II-A became marked, and, in some parts, all the deformative acini were found to be necrotic. This necrosis might be attributed to the insufficient blood supply caused by the increase of collagenous fibers, and by the consequent liver cirrhosis.

Cholangiofibrosis was found in 8 cases. In these cases, thick ducts of the adenomatous structure, accompanied with innumerable thin bile ducts, covered a comparatively large area. Usually, the cells which composed the ducts formed a layer of flat, cuboidal, or columnal epithelia, but, sometimes, it was found that they were rich in chromatin and proliferated to form stratified epithelia. PAS-positive substances were found in almost every duct.

The increase of collagenous fibers, as mentioned above, was marked at this stage. It was found mainly in the area of cholangiofibrosis, and also around the thin ducts, forming an extremely irregular network. Necrosis, which was often found either in a comparatively large area of the liver parenchyma or in the area of cholangiofibrosis, seemed to bear some relation to the deficient circulation caused by the increase of collagenous fibers. In 10 cases, liver cysts were noticed. These cysts seemed to bear some relation to the increase of collagenous fibers.

V) Five months of DAB-feeding (Group V-A): A remarkable liver cell proliferation took place. Adenomatous hyperplasia was found in 13 cases, and nodular hyperplasia in 15 cases. A typical adenoma was found in 3 cases (Fig. 6). Eight rats showed the formation of intensive cholangiofibrosis, while cyst formation was found in as many as 12 rats.

In the same manner as mentioned above, deep violet-stained substances could be found, by means of PAS-staining, in the proliferated epithelia of the portal tracts and also in the cavities of them. But a specific character is that these substances could not be found at all in the areas of proliferating liver cells, such as, of the adenomatous hyperplasia or the adenoma.

B) FINDINGS IN THE LIVER OF THE RATS SACRIFICED OR DIED AFTER THE 210TH EXPERIMENTAL DAY.

The animal experiment was continued for 420 days. The first liver tumor was found in a rat belonging to Group V, when it was sacrificed on the 210th experimental day. Therefore, all the rats which survived longer than 210 days were regarded as the valid instances to calculate the formation rate of liver cancers. Among the rats which died during the period between the 210th and 420th experimental days, most of those in Groups I, II, and III died of

Table 3. Incidence of adenomatous hyperplasia in the liver.

Group	Immed. after the feed.	Months after the beginning of the DAB-feeding −8	−9	−10	−11	−12	−13	−14	at 420 days	Total
I-A	0/11	—	—	—	—	—	—	—	—	0/11
B	—	—	—	—	—	—	—	0/1	0/9	0/10
C	—	0/1	0/1	0/2	—	0/1	0/1	0/2	0/12	0/20
II-A	0/10	—	—	—	—	—	—	—	—	0/10
B	—	0/1	—	—	—	—	—	—	0/10	0/11
C	—	—	0/1	0/1	—	—	—	0/2	3/14	3/18
III-A	0/16	—	—	—	—	—	—	—	—	0/16
B	—	—	0/1	0/1	—	—	—	0/2	1/9	1/13
C	—	0/1	0/1	—	—	—	1/1	0/1	1/16	2/20
IV-A	7/12	—	—	—	—	—	—	—	—	7/12
B	—	2/3	0/2	—	0/1	0/1	1/2	—	1/7	4/15
C	—	1/4	0/1	—	1/2	—	1/3	1/1	4/15	9/26
V-A	13/17	—	—	—	—	—	—	—	—	13/17
B	—	1/1	1/3	0/4	3/4	—	—	0/1	0/13	5/26
C	—	0/6	4/7	0/1	—	2/2	0/1	1/2	3/10	10/29

Table 4. Incidence of adenoma in the liver.

Group	Immed. after the feed.	Months after the beginning of the DAB-feeding −8	−9	−10	−11	−12	−13	−14	at 420 days	Total
I-A	0/11	—	—	—	—	—	—	—	—	0/11
B	—	—	—	—	—	—	—	0/1	0/9	0/10
C	—	0/1	0/1	0/2	—	0/1	0/1	0/2	0/12	0/20
II-A	0/10	—	—	—	—	—	—	—	—	0/10
B	—	0/1	—	—	—	—	—	—	0/10	0/11
C	—	0/1	0/1	—	—	—	—	0/2	2/14	2/18
III-A	0/16	—	—	—	—	—	—	—	—	0/16
B	—	—	—	0/1	0/1	—	—	0/2	0/9	0/13
C	—	0/1	0/1	—	—	—	1/1	0/1	2/16	3/20
IV-A	0/12	—	—	—	—	—	—	—	—	0/12
B	—	0/3	0/1	—	0/1	0/1	1/2	—	2/7	3/15
C	—	0/4	0/1	—	1/2	—	1/3	0/1	5/15	7/26
V-A	3/17	—	—	—	—	—	—	—	—	3/17
B	—	1/1	2/3	1/4	0/4	—	—	0/1	2/13	6/26
C	—	0/6	3/7	0/1	—	2/2	0/1	1/2	1/10	7/29

enterocolitis or pneumonia, and most of those in Groups IV and V died of liver cancers.

The relation between the experimental months and the incidence of the changes in the liver such as the development rate of adenomatous hyperplasia (Table 3), adenomas (Table 4), malignant liver tumors (Table 5), bile duct proliferation (Table 6), cholangiofibrosis (Table 7) and liver cysts (Table 8) was shown in each table. The average liver weight and their index of the animals in which no liver tumor was found, were tabulated in Table 9.

Table 5. Incidence of liver cancers.

Group	Immed. after the feed.	Months after the beginning of the DAB-feeding −8	−9	−10	−11	−12	−13	−14	at 420 days	Total
I-A	0/11	—	—	—	—	—	—	—	—	0/11
B	—	—	—	—	—	—	—	0/1	0/9	0/10
C	—	0/1	0/1	0/2	—	0/1	0/1	0/2	0/12	0/20
II-A	0/10	—	—	—	—	—	—	—	—	0/10
B	—	0/1	—	—	—	—	—	—	0/10	0/11
C	—	—	0/1	0/1	—	—	—	0/2	0/14	0/18
III-A	0/16	—	—	—	—	—	—	—	—	0/16
B	—	—	0/1	0/1	—	—	—	0/2	0/9	0/13
C	—	1/1	0/1	—	—	—	1/1	1/1	2/16	5/20
IV-A	0/12	—	—	—	—	—	—	—	—	0/12
B	—	1/3	0/1	—	0/1	1/1	2/2	—	0/7	4/15
C	—	0/4	1/1	—	2/2	—	3/3	0/1	2/15	8/26
V-A	0/17	—	—	—	—	—	—	—	—	0/17
B	—	1/1	3/3	2/4	4/4	—	—	0/1	0/13	10/26
C	—	5/6	7/7	0/1	—	2/2	1/1	1/2	2/10	18/29

Table 6. Incidence of bile ducts proliferation in the liver.

Group	Immed. after the feed.	Months after the beginning of the DAB-feeding −8	−9	−10	−11	−12	−13	−14	at 420 days	Total
I-A	11/11	—	—	—	—	—	—	—	—	11/11
B	—	—	—	—	—	—	—	1/1	0/9	1/10
C	—	1/1	0/1	2/2	—	0/1	1/1	1/2	7/12	12/20
II-A	10/10	—	—	—	—	—	—	—	—	10/10
B	—	1/1	—	—	—	—	—	—	3/10	4/11
C	—	—	1/1	1/1	—	—	—	1/2	14/14	17/18
III-A	16/16	—	—	—	—	—	—	—	—	16/16
B	—	—	1/1	0/1	—	—	—	1/2	7/9	9/13
C	—	1/1	0/1	—	—	—	1/1	1/1	16/16	19/20
IV-A	12/12	—	—	—	—	—	—	—	—	12/12
B	—	3/3	1/1	—	1/1	1/1	2/2	—	7/7	15/15
C	—	4/4	1/1	—	1/2	—	1/3	0/1	15/15	22/26
V-A	17/17	—	—	—	—	—	—	—	—	17/17
B	—	1/1	3/3	4/4	4/4	—	—	1/1	10/13	23/26
C	—	6/6	7/7	1/1	—	2/2	1/1	2/2	9/10	28/29

The histological classification of developed liver tumors by means of DAB-feeding, has been made by many investigators[15),16)] from various points of view, and its standard is unsettled. In this experiment, tumors were divided into 4 types by the following criteria: Type I; typical hepatoma; the cancers of this type have the parenchyma that consists of cord-like or partially adenomatous cell clusters, of which each cell has a comparatively large nucleus lacking in chromatin, and has a comparatively large cell bodies. In most cancers of this type, cell cords

Table 7. Incidence of cholangiofibrosis formation in the liver.

Group	Immed. after the feed.	Months after the beginning of the DAB-feeding −8	−9	−10	−11	−12	−13	−14	at 420 days	Total
I-A	0/11	—	—	—	—	—	—	—	—	0/11
B	—	—	—	—	—	—	—	0/1	0/9	0/10
C	—	0/1	0/1	0/2	—	0/1	0/1	0/2	0/12	0/20
II-A	4/10	—	—	—	—	—	—	—	—	4/10
B	—	0/1	—	—	—	—	—	—	0/10	0/11
C	—	—	1/1	0/1	—	—	—	0/2	5/14	6/18
III-A	3/16	—	—	—	—	—	—	—	—	3/16
B	—	—	0/1	0/1	—	—	—	0/2	2/9	2/13
C	—	1/1	0/1	—	—	—	0/1	0/1	1/16	2/20
IV-A	8/12	—	—	—	—	—	—	—	—	8/12
B	—	2/3	0/1	—	1/1	1/1	2/2	—	1/7	7/15
C	—	4/4	1/1	—	2/2	—	1/3	1/1	5/15	14/26
V-A	8/17	—	—	—	—	—	—	—	—	8/17
B	—	1/1	1/3	2/4	2/4	—	—	0/1	13/13	19/26
C	—	3/6	4/7	0/1	—	0/2	1/1	0/2	8/10	16/29

Table 8. Incidence of cyst formation in the liver.

Group	Immed. after the feed.	Months after the beginning of the DAB-feeding −8	−9	−10	−11	−12	−13	−14	at 420 days	Total
I-A	1/11	—	—	—	—	—	—	—	—	1/11
B	—	—	—	—	—	—	—	0/1	1/9	1/10
C	—	1/1	0/1	0/2	—	0/1	1/1	1/2	7/12	10/20
II-A	2/10	—	—	—	—	—	—	—	—	2/10
B	—	0/1	—	—	—	—	—	—	3/10	3/11
C	—	—	1/1	1/1	—	—	—	1/2	14/14	16/18
III-A	3/16	—	—	—	—	—	—	—	—	3/16
B	—	—	1/1	0/1	—	—	—	0/2	9/9	10/13
C	—	0/1	1/1	—	—	—	1/1	1/1	13/16	16/20
IV-A	10/12	—	—	—	—	—	—	—	—	10/12
B	—	2/3	1/1	—	1/1	1/1	2/2	—	7/7	14/15
C	—	4/4	1/1	—	2/2	—	3/3	1/1	15/15	26/26
V-A	12/17	—	—	—	—	—	—	—	—	12/17
B	—	1/1	3/3	4/4	4/4	—	—	1/1	12/13	25/26
C	—	3/6	6/7	1/1	—	1/2	1/1	1/2	10/10	23/29

and blood capillaries meet directly or with only an endothelium between them. The fibrous stroma are found very little. The cancers of this type do not contain PAS-positive substances at all. (Fig. 7). Type II; (1) The cancers of this type have a histological structure quite similar to that of the cancers of Type I. The stainability of each cell by H-E staining is also similar to that of Type I. But the tumors have PAS-positive substances in the cytoplasms, although such cases were very few (only 2 in all). (Fig. 8). (2) The cancers of this group

Table 9. Average liver weight and index of the animals in which the liver tumors were not detected.

Group	Animals	Liver weight (min.-max.)	Liver index (min.-max.)
I-B	10	11.6 gr. (8.3-16.5)	4.9 (4.1-6.4)
C	20	13.5 gr. (10.4-25.8)	5.3 (4.0-9.4)
II-B	11	11.5 gr. (7.4-14.7)	5.2 (4.1-6.2)
C	16	16.3 gr. (8.1-35.0)	6.4 (4.5-11.5)
III-B	13	15.0 gr. (9.4-23.6)	5.5 (4.9-7.3)
C	15	13.6 gr. (9.5-16.8)	5.8 (4.6-6.6)
IV-B	9	14.1 gr. (11.3-19.9)	6.1 (4.7-8.3)
C	15	16.5 gr. (9.7-53.2)	6.3 (5.1-17.7)
V-B	14	14.7 gr. (8.9-26.1)	6.9 (4.0-11.1)
C	11	15.2 gr. (11.3-21.3)	6.6 (5.0-8.6)

keep, as a whole, an adenomatous arrangement of the cells, and some collagenous fibers are found around those structures (Fig. 9). Some parenchymal cells are similar to those of Type I, and some take the form of a columnal epithelium similar to globlet cells, but both of them have PAS-positive substances in the cytoplasms and ducts. Judging from the state of the arrangement of cells and findings in PAS-staining, some of these cancers seem to be type of cholangiocarcinoma, however judging from the fact that they have little stroma, and that the stainability of their parenchymal cells by means of H-E staining is similar to that of liver cells, these cancers may be considered to be of an intermediate type. Some of the cancers of this group showed bone or cartilage formation (Fig. 10) and squamous cell metaplasia (Fig. 11), just as those of Type III. Type III ; cholangiocarcinoma ; in the cancers of this type, intensive duct formation of parenchymal cells and considerable proliferation of connective tissues as stroma are found (Fig. 12). Such findings are the same with those in human cholangiocarcinoma. These cancers have a great deal of PAS-positive substances in the cytoplasms and in the ducts. Type IV ; the cancers of this type are those which do not belong to any types mentioned above, and they are composed of the small nest of epithelial cells (Fig. 13). This is what is called the type of carcinoma simplex.

The developed tumors found in the rats of each group were classified into the above 4 types and tabulated in Table 10.

Table 10. Histological classification of the induced tumors.

Group	Histological types I	II	III	IV
III-B	—	—	—	—
C	3/5=60%	2/5=40%	—	—
IV-B	4/4=100%	3/4=75%	2/4=50%	1/4=25%
C	8/8=100%	1/8=13%	1/8=13%	—
V-B	10/10=100%	5/10=50%	3/10=30%	1/10=10%
C	18/18=100%	13/18=72%	3/18=17%	2/18=11%

1) Group I. a) One month of DAB-feeding (Group I-B) : One of the rats died of pneumonia on the 402 nd experimental day, and the other 9 were sacrificed on 420 th day. Histological examination showed that, in all the cases, there was a

conspicuous dilatation of both central veins and the intralobular network of blood capillaries, but the structure of liver acini was not in disorder. In the liver cells, only the slight pycnosis of nuclei and atrophy of cell cords could be noticed, and there was no other conspicuous change (Fig. 14). It was observed that such interlobular proliferation of bile ducts as clearly found immediately after 1 month of DAB-feeding became very weak at this time; that there remained a few atrophic bile ducts: and the spot was edematous and stained light blue by Azan-staining. Liver cyst formation was found in one case.

b) One month of DAB-feeding followed by 8 months of twice weekly MC-painting (Group I-C): Eight rats did mainly of pneumonia between the 240 th and 420 th day, and the other 12 rats were sacrificed on the 420 th day. All the rats showed such a distinct dilatation of both central veins and the interlobular network of blood capillaries as was found in the control rats, and 12 of them showed the intralobular proliferation of bile ducts. In 7 cases out of these 12, PAS-positive substances were found in the ducts. The formation of unilocular bile duct cysts was found in 10 rats, and the wall of the cysts was composed of a layer of flat, cuboidal or columnal epithelia. In the liver cells, the diversity of cells in size and the pycnosis of nuclei were observed, but the grade of these changes was low, and no proliferating features were found at all.

II) Group II. Two months of DAB-feeding (Group II-B): One of the animals died of pneumonia on the 240 th day, and the others were sacrificed on the 420 th day. The findings of hepatic cell cords were not so different from those in the animals in Group I-B, and a hyperplastic figure could not be found. A slight increase of bile ducts was found in 4 rats, but the strong intraacinary proliferation could not be found this time which was observed in the liver of the rats killed immediately after the stop of the feeding (Group II-A). The spaces between the acini were somewhat wide and edematous, stained homogeniously light blue by means of Azan-staining, and there were atrophic bile canaliculi. The formation of unilocular bile duct cysts was found in 3 cases.

b) Two months of DAB-feeding followed by 7 months of MC-painting (Group II-C): Four of the animals died between the 240 th and 420 th day, and the other 14 rats were sacrificed on the 420 th day. The findings different from those of the animals of Group II-B, were that cholangiofibrosis was still existent and adenomas were formed.

The increase of bile canaliculi was slow in comparison with that which was seen in the rats immediately after the stop of the feeding (Group II-A), but it was still found in the intralobular region, and, in 17 cases, the lobulus was found to be divided into many irregular parts. The marked cholangiofibrosis was found in 6 cases. The structure of cholangiofibrosis was slightly differed from that which

was found in the liver of Group II-A, in which the proliferation of the bile ducts themselves was a little slow and collagenization was remarkable. By means of Azan-staining, vivid collagenous fibers could be demonstrated around each duct.

In all the cases but 2, cyst formation was found in a very remarkable degree. In 3 cases, the cells of the cyst wall were abundant in chromatin and proliferated papillomatously into the glandular cavity presenting multi-locular appearance. In one of these cases, the liver was 35 g in weight and its index was 11.5.

With regard to the proliferating changes of hepatic cells, one case showed the adenomatous hyperplasia that corresponded to that which was found in the liver of the rats immediately after 4 months of DAB-feeding (Group IV-A), and another 2 cases showed the nodules of adenoma as well as adenomatous hyperplasia. In one of the 2 cases, the adenoma was bean-sized and showed the figure of typical adenoma. In the other case, the liver was 23.4 g in weight, its index was 11.1, and the tumor nodule was as big as the tip of the thumb. In this case, the adenoma took the shape of bunch composed of several small adenoma nodules, of which the parenchymal cells were, histologically, somewhat different from each other, and each nodule was separated by a little collagenous fiber surrounding it. At a peripheral part of the adenoma, it was found that the cell cord without endothelium irregularly proliferated to form some layer, and that the polymorphysm of cells was developed in high degree, so that a small part of the lesion presented a figure which could be hardly distinguished from malignant hepatoma (Figs. 15, 16, and 17).

III) Group III. a) Three months of DAB-feeding (Group III-B): Four of the rats died between the 270 th and the 420 th day, and the other 9 were sacrificed on the 420 th day. The inter- and intralobular proliferation of bile ducts observed in the rats of Group III-A became much less in its degree, and the acini retrieved comparatively regular arrangement, but the slight increase of bile canaliculi inside the acinus could be observed in 9 cases. Two of those cases showed cholangiofibrosis at the spot of Glisson's capsule and the presence of PAS-positive substances.

In all the cases but one, the hepatic cells exhibited no such adenomatous hyperplasia as was found in rats of Group IV-A. A slight dilatation of central veins and sinuses was observed representing a slight flection of liver cell cords.

b) Three months of DAB-feeding followed by 6 months of MC-painting (Group III-C): Four of the rats died between the 240 th and 420 th day, and 3 of those cases developed liver cancers. Among 16 survivors sacrificed on the 420 th day, liver cancer was detected in 2 cases.

The most characteristic phenomenon in this group was that there were 5 cases in which development of liver cancers was found. Thus, for example, in a case sacrificed on the 212 th day, development of a soft hemorrhagic tumor as large as

a ping-pong ball was found in the liver besides cholangiofibrosis and cyst formation. Several metastatic nodules which were smaller than or as large as bean-sized, were found in the omentum and the paragenital tissue of this rat. Histologically, the both main and metastatic nodules were of Type II. The liver cancers found in 3 cases out of the other 4 were of Type I, and that in the remaining case was of Type II. In addition, an adenoma larger than a bean in size was found in each liver of those rats which bore a tumor belonging to Type I, and in one of them, nodules of adenomatous hyperplasia were found additionally. It was only in the above 3 cases that adenoma formation was detected. But, regarding adenomatous hyperplasia, the above one and another one showed its existence. Besides, each liver of those 15 cases, in which tumors were not developed, showed a proliferating area which was similar to nodular hyperplasia.

Although the degree of increase of bile canaliculi was less than that which was observed in the liver of Group III-A, the proliferated bile canaliculi were found still in as many as 19 cases, 3 of which showed formation of cholangiofibrosis at the spot of Glisson's capsule. Moreover, cyst formation was found in 16 cases, 3 of which showed that the cells composing the cyst wall increased their stainability and proliferated papillomatously in the cavities of the cyst.

IV) Group IV. a) Four months of DAB-feeding (Group IV-B): Eight rats died during the 240 th and 390 th day, and in 4 of those cases the cause of their death was the formation of liver cancers. The 7 survivors were sacrificed on the 420 th day.

Hyperplasia and proliferation of hepatic cells were seen in 9 cases, and in 4 cases of which the features were adenomatous. Other 3 cases of those 9 showed that each liver had one nodule as large as the tip of the little finger, and histologically the nodule was an adenoma. Malignant liver tumors were observed in 4 cases. Histologically, each of these 4 cases showed that some of those tumor nodules belonged to Type I and others to Type II. In 2 of these 4 cases, a typical cholangiocarcinoma was found.

In all cases the interlobular zone was slightly edematous, and there were somewhat atrophic bile canaliculi still in existence. Seven cases showed an area of cholangiofibrosis comparatively abundant in collagenous fibers. Cyst formation was found in 14 rats, 2 of which showed that cysts were multi-locular and induced a remarkable proliferation of wall cells.

b) Four months of DAB-feeding followed by 5 months of MC-painting (Group IV-C): Eleven rats died by the 420 th day, of which 6 died from liver cancer. The 15 survivors were sacrificed on the 420 th day.

There were no findings different in quality from those of the rats of Group IV-B, but the proliferation of both hepatic and bile duct cells was stronger; that

is, a structure which corresponded to the nodular hyperplasia observed in the rats of Group III-A, was seen in 24 cases, and the adenomatous structure was found in 9 cases out of those 24. Typical adenoma formation was noticed in 7 cases. Liver cancers were found in 8 rats and 3 of those had a nodule of typical adenoma in other liver lobes. Histologically, 7 cases of those 8 liver cancers belonged to Type I, and the remaining to Types I, II, and III.

A distinct bile duct proliferation and formation of cholangiofibrosis were revealed stronger than those which were seen in the rats of Group IV-A. Cholangiofibrosis was found in 14 cases. Cyst formation was found in all the cases, one of which showed that all the lobes were covered with cysts, and that the liver was 53.2 g in weight and its index reached 17.7. In 4 cases, the cells of the cyst wall proliferated into the cavity and the cysts were found to be multi-locular.

V) Group V. a) Five months of DAB-feeding (Group V-B): Thirteen rats, including 10 cases of tumor death, died during the 240 th and 420 th day, and the 13 survivors were sacrificed on the 420 th day. Development of liver cancers could not be found at any of the sacrificed animals.

Slight proliferating changes of hepatic cells were found in 17 cases, and formation of adenomatous hyperplasia in 5 cases. Six cases showed typical adenomas, and 10 cases malignant liver tumors. Histologically, in 3 cases, the liver cancers were composed only of typical hepatomas, in other 4 case they were composed of typical ones and those belonging to Type II, and in other 2 cases of both typical ones and cholangiocarcinomas, and in the remaining case of Types I, II, III, and IV.

Increase of bile ducts was observed in 23 cases, and 19 of which showed formation of cholangiofibrosis. However, in comparison with the animals of Group V-A, those 23 cases showed not so intensive proliferation of bile canaliculi, and many of the ducts surrounded by the collagenous fibers were found to be a little atrophic, which was the same as in the other rats of subgroup B. In contrast to those findings, cyst formation showed much more augmentation than in the case just after the stop of the DAB-feeding; and it was found in as many as 25 animals. Especially in 2 of them, the cell proliferation of the cyst wall was so strong and the microscopical cysts were so massed, taking the shape of a macroscopically someweat translucent white nodule sweeled up on the cutting surface, that the appearance seemed to be that of a nodule of an adenoma or small hepatoma (Fig. 18). The liver of one of those 2 rats was 26.1 g in weight, and its index was 11.1.

b) Five months of DAB-feeding followed by 4 months of MC-painting (Group V-C): Sixteen rats died from the development of liver cancers by the 420 th day, and other 3 animals of pneumonia. The 10 surviving rats were sacrificed on the

420 th day, 2 of which revealed development of liver cancers.

Liver cancers were found, as mentioned above, in 18 rats, of which 3 bore only typical hepatomas, 10 both typical ones and cancers of Type II, 3 cancers of Types I, II, and III, and other 2 both of Types I and IV. Six of those 18 rats revealed adenoma nodules as well as liver cancers. Adenomas unaccompanied with liver cancers were found in only one case. Small nodules which exhibited the structure of adenomatous hyperplasia were found in 10 animals, of which 8 cases showed adenomas and or hepatomas in other liver lobes. Nodular hyperplasia was found in as many as 23 cases.

The grade of proliferation of the bile duct system, was not so distinct as that observed just after the stop of DAB-feeding, but the proliferation was still found in the somewhat edematous stromas which were scattered in other area than the normal Glisson's capsule, and the number of these cases reached 28. Moreover, the picture of cholangiofibrosis abundant in collagenous fibers was seen in 16 cases, and cyst formation was found in 23 cases.

Throughout the whole groups, the tumors developed in other organs or tissues than the liver were observed as follows: 3, 1, and 1 cases of squamous cell carcinoma were found in Groups I, IV, and V respectively; and 8 and 7 cases of skin papillomas were found in Groups I and II respectively. These tumors were found only in the MC-painted areas of the skin. In addition, 2 cases of breast tumors were recognized in the control and in the experimental animals, and each of those tumors showed, histologically, the picture of fibroadenoma mammae. These were the same as the spontaneous tumors developed in old rats without any treatments.

C) FORMATION OF METASTASIS AND TRANSPLANTABILITY OF THE INDUCED TUMORS.

In the present study, the host animals bearing the liver tumors were sacrificed when they reached agonal condition and the frequency of metastasis was examined as a criterion of the malignancy of developed tumors. The intra-abdominal tissues, lungs, and mediastinal lymph nodes were examined for existence of metastatic nodules. The results were tabulated in Table 11. It is concluded generally that the longer the term of DAB-feeding was the higher the frequency of metastasis become, while there was no definite correlation between influence of MC-painting

Table 11. Formation of metastasis in the primary tumor rats.

Group	Intraperitoneal tissues	Lungs	Mediastinal lymph nodes
III-B	—	—	—
C	1/5=20%	0/5=0%	0/5=0%
IV-B	2/4=50%	1/4=25%	0/4=0%
C	4/8=50%	3/8=38%	2/8=25%
V-B	8/10=80%	4/10=40%	3/10=30%
C	12/18=67%	8/18=44%	2/18=11%

and frequency of metastasis.

As for the transplantation of primary tumor nodules and/or tumor ascites to the intraperitoneal cavity of normal rats, the results in the first transfer generation were indicated in Table 12. Transplantation rate was higher in the tumors

Table 12. Transplantation rate of the primary tumors and tumor ascites.

Group	Result	Total
III-B	—	0/2=0%
C	0/2=0%	
IV-B	1/4=25%	1/9=11%
C	0/5=0%	
V-B	2/8=25%	6/19=32%
C	4/11=36%	

which developed in the animals given a longer term of DAB-feeding. But the influence of MC-painting for the increase of transplantability was not clearly demonstrated.

The above-mentioned results of transplantation are not so much different from the results of the experiments having been conducted for the purpose of researching the ascitic conversion of azodye-induced liver cancers. The details of the experiments were presented in the GANN, Vol. 49, Suppl. p. 191, 1958.

Discussion

I) REGARDING THE STOP EXPERIMENT OF DAB-FEEDING

In the present experiments, effect of the stop of DAB-feeding (subgroup B) as well as the influence of MC-treatment (subgroup C) after the stop of DAB-feeding were examined.

Close attention was paid to uncover the kind of histological changes of the liver caused by DAB-feeding that can progress to the liver cancers, after discontinuation of the feedings. Results showed that liver cancers failed to develop in subgroup B of Groups I, II, and III, but developed in Groups IV and V; therefore, the changes which were first noticed after 4 months of DAB-feeding attracted the greatest interest.

The changes of hepatic cells caused by DAB-feeding are divided into reversible and irreversible changes, by means of comparative studies of the liver of the rats which were sacrificed immediately after the stop of the DAB-feeding (subgroup A) and of those which survived more than 210 days (subgroup B). The results so far obtained were as follows: For example, the variety in size, division and enlargement of hepatic cells, observed in the rats by the end of the feeding period of 2 months, as well as the slightly degenerating changes observed at the same time were reversible but some of the proliferating lesions of massing hepatic cells such

as observed in the liver after 3 months of DAB-feeding remained in the same state till about one year···the state in which the cells were comparatively small and possessed a high nucleo-cytoplasmic ratio, and the acini were so much filled with cell cords that the intralobular network of blood capillaries could hardly be observed; that is say, nodular hyperplasia. Furthermore, after 4 months of the feeding, there was observed a stage of adenomatous hyperplasia, where the cytoplasms of proliferated liver cells can be stained a little basophilic, and the cell cords develop forming some layers, and compressing or entering into the surrounding cell cords. When they came to stage of adenomatous hyperplasia, not only those cell cords remained in the same state and were irreversible, but also some of them appeared to have the potentiality of developing, through adenoms, into liver cancers. This may be accepted from the fact that the above-mentioned picture of the liver was observed for the first time in 7 out of 12 rats just after 4 months of the DAB-feeding (Group IV-A), and that liver cancers were also developed for the first time in 4 out of 15 animals of this group (Group IV-B) as seen in Table 13.

Table 13. Correlation between the incidence of adenomatous hyperplasia, adenoma, and liver cancers by means of the DAB stop-examination.

	Adenomatous hyperplasia		Adenoma	Liver cancers
Group	Subgroup A	Subgroup B		
I	0/11=0%	5/10=0%	0/10=0%	0/10=0%
II	0/10=0%	0/11=0%	0/11=0%	0/11=0%
III	0/16=0%	1/13=8%	0/13=0%	0/13=0%
IV	7/12=58%	4/15=27%	3/15=20%	4/15=27%
V	13/17=76%	5/26=19%	6/26=23%	10/26=39%

The necessary feeding term and doses of DAB for the formation of adenomatous hyperplasia seemed to be greatly dependent on the condition of experiment. On this subject, there have been many reports stating that the average interval of the liver cancer incidence and the formation rate of cancers considerably varied depending on the components of a basic diet.[17),18)] In the present experiment, the mortality was low, but the formation rate of liver cancers was also low and the average interval was remarkably long, in comparison with the usual experiments conducted by using unpolished rice to which an oil solution of DAB was added. The same tendency was noticed in another series of experiments in which the same DAB-diet was given for 8 months.[19)] The tendency may be considered to be caused by the components of the basic diet···especially by high calorie and high protein···but the conditions of absorption must also be taken into consideration; for, in this diet, as crystallized DAB was used···to be concrete, crystallized DAB was dissolved in ethanol, mixed with the powdered basic diet and excipient, and made into tablets···, it seems that an absorbed dose of DAB itself may be different from when DAB was dissolved in oil. The details concerning the experiments on the absorption of DAB, using albino rats and dogs, will be published in the near feature by Dr. Y. Hashimoto, University of Tokyo.

The relation of the period of DAB-feedings to the formation rate of liver cancers and the average interval of the liver cancer development was tabulated in Table 5, which showed a tendency that the longer the feeding term of a group was, the higher the formation rate and the shorter the average interval became. This result was the same as those which have been reported by W. Lauber.[20]

In the next place, the relation between the histo-pathological classification of induced tumors and the term of DAB-feeding was indicated in Table 10. It was not always possible, as a matter of fact, to decide the origins of the induced tumors morphologically, namely the hepatic cell origin and the bile duct cell origin, so that those tumors were classified in Type II. The reason why they were not classified in Type I was that their duct formation was distinct, and that they possessed PAS-staining positive substances after the glycogen digestion in the cavities and cytoplasms. Moreover, the reason they were not classified in Type III was that the quantity of the collagenous fibers surrounding the ducts was not very large, and that the cells composing the ducts showed, by means of H-E staining, to have appearances more similar to hepatic cells than to bile duct cells. As it was found in many cases, however, that the liver tumors of Types I and II, or Types II and III, existed together even in the same tumor nodules without any line of distinction, it was considered that the features of the tumors might be convertible to each other. As the result of the classification according to the above criteria, the appearance of cholangiocarcinoma was seen more often in Group IV which had a shorter term of the feeding than in Group V. Similar result was reported by Lauber and Danneberg.[20] But, the type of cholangiocarcinoma was not seen at all in the experimental rats of Group III-C. Furthermore, in other series of experiment[19] in which the term of DAB-feeding was 8 months, the formation rate of cholangiocarcinoma was so high that it was impossible to draw any conclusion about the relation between the doses of DAB-feeding and the histological classification of the induced tumors.

The proliferation of bile capillaries and the formation of cholangiofibrosis were indicated in Tables 6 and 7 respectively. The proliferation of bile ducts in the liver was observed intensively in the rats sacrificed just after the stop of the feeding, but it gradually became atrophic afterwards, and the proliferation of collagenous fibers appeared to show a strong tendency towards forming cicatrices. Similar findings were described by Lauber. Regarding the degree of those changes, the bile duct proliferated by the end of 3 months of the feeding became remarkably atrophic, but these which were proliferated after more than 3 months, that is, after the beginning of collagenization were still considerably in existence.

W. Lauber and others[20] concluded that cyst formation was not correlated with the term of DAB-feeding, but the longer the survival days of the rats after the

stop of the feeding were, the more often cyst formation appeared. But the present result seemed not always to correspond with their's. Thus, for example, as indicated in Table 8, in the groups given less than 3 months of DAB-feeding, the longer the feeding term was, the more often cyst formation appeared, but in the groups given more than 4 months of DAB-feeding, cyst formation was observed in almost all the animals. Regarding the mechanism of cyst formation, the histogenesis suggested by Lauber et al., seemed to be acceptable, but there were some findings in which the cells themselves that composed the cyst wall showed a neoplastic proliferation.

II) REGARDING THE INFLUENCE OF MC-PAINTING.

Many researches have admitted that, percutaneous administration of MC on normal rats induced neither proliferation of hepatic cells, nor development of liver adenomas and malignant hepatomas. In our other experiment, 0.3% MC solution in acetone was twice weekly painted for 1 to 5 months on the skin of normal rats, but no proliferating changes of liver cells were found. When the same treatment was given to mice for 15 weeks, or when the subcutaneous injection of 1 mg of MC was applied to mice,[21),22)] skin carcinomas and subcutaneous sarcomas developed but no proliferative changes of liver cells could be detected.

In the present experiment in which MC-treatment was given after the stop of DAB-feeding, however, 2 cases of liver adenoma were found in Group II-C, while no adenomas developed in the control rats of Group II-B. In one of these cases, the adenoma was as large as the thumb-tip size and possessed an area where typical growth was marked and the feature could hardly be distinguished from a hepatoma. Furthermore, there were obtained 5 cases of liver cancers and 3 cases of adenomas in the experimental rats of Group III-C, while the control animals (Group III-B) failed to show either adenomas or liver cancers. These results might be interpreted as follows: The liver cells which were affected by DAB-feeding, were changed initially in their morphological and biological characteristics, but did not possess enough potency to develop themselves and form a neoplasm. These initial changes could be promoted by the action of MC-painting, inducing the neoplastic conversion of the affected liver cells.

This interpretation that MC-treatment might have such an action can be supported by the fact that the revelation rate of nodular and adenomatous hyperplasia was increased in experimental rats of Groups II and III, compared with that of controls. Thus, for example, in control rats examined nearly one year after the stop of the DAB-feeding, such a picture of nodular hyperplasia found immediately after 3 months of the feeding, were not found in Group II-B and only found in 8 out of 13 cases of Group III-B; while, in regard to the livers of the MC-painted rats, those nodules were found in 4 out of 18 cases of Group II-C and in 16 out

of 20 cases of Group III–C.

Moreover, the formation rate of liver cancers was a little higher, and the average interval of the liver cancer incidence was shorter in the cases of Group IV–C and V–C than in the cases of the control rats without the MC–treatment. These differences were quite distinct in Group V, which was given 5 months of DAB–feeding. These results are very interesting in comparison with those in the report that the formation rate becomes low and the average interval long when rats were given DAB–feeding simultaneously with MC.[23]

The carcinogenic process of liver cells initiated by DAB was more marked when DAB–feeding was followed by MC–painting, compared with in the groups without MC–painting, as stated above. But if the term of MC–treatment was replaced by the same period of an additional DAB–feeding, development of liver cancers increased markedly. That is to say, if the formation rate of liver cancers of the control rats in Group V–B, which have been fed with DAB for 5 months, is compared with the experimental animals in Groups I–C, II–C, III–C, and IV–C, in which DAB–feeding was replaced by MC–treatment in the middle, the tumor incidence is very smaller in the painted groups in which the total period of treatment reached as long as 9 months, than in the group only given 5 months of DAB–feeding. The result seems to show that MC–painting has a less potent influence on hapatic carcinogenesis than continuous DAB–feeding.

The effects of MC–painting on the changes of bile ducts caused by DAB–feeding were as follows: The proliferation of bile canaliculi by DAB and the lesions of cholangiofibrosis were marked in the rats Groups I–C and II–C compared with the control animals (Groups I–B and II–B), while such a difference was not so distinct in Groups III, IV, and V (Tables 6 and 7). The cyst formation was remarkable in the experimental rats of Groups I–C, II–C, and III–C, when compared with that of the control, but in Groups IV and V, as the cyst formation was found in almost all the controls, there was no conspicuous differences between them. The difference seen in Groups I, II, and III might be attributed to the action of MC–painting, which either prevent the regression of the proliferated bile ducts or promote the proliferation of them.

One of the interesting problems suggested through this series of experiments, is to compare the relation between the findings in this study and in the two-phase mechanism of skin carcinogenesis in mice or rabbits observed by Berenblum,[1] Rous,[2] etc. For this purpose, however, further experiment have to be carried out under several conditions such as; (1) various intervals between the end of DAB–feeding given as initiating stimuli and the beginning of MC–treatment given as promoting stimuli; (2) the formation rate of liver cancers and the average interval of the liver cancer incidence in case of MC–painting followed by DAB–feeding; (3) the

various kinds of irritants that are capable of acting as initiators or promotors; etc. However, the results of the present experiment suggest that the hepatic cell changes, initiated by a short time of DAB-feeding but unable to develop by themselves to form neoplasms, can surely be developed, by means of MC-treatment, to form neoplasms—liver adenomas and liver cancers. In this sense, these results may be considered to support the concept on the two-phase mechanism.

However, comparing this experiment with Berenblum's, there remain such a problem as the following: what kind of changes in the process of azodye carcinogenesis is equivalent to skin papillomas, as criteria to judge "a tumor has been induced". If both cases were compared only by means of their potency of autonomous proliferation, the stage of skin papillomas seems to be equivalent to that of adenomatous hyperplasia in DAB-carcinogenesis. For, it was reported that, if the promoting stimuli were stopped, some of the skin papillomas showed spontaneous regression and some remained in the same status, while some continued growing and showed a malignant conversion: and these results seemed to be equivalent to the termination of adenomatous hyperplasia in Group IV-A, which was put under DAB stop-examination. And if its formation should be regarded as a criterion, it would be accompanied with great technical difficulties to conduct the experiments. Moreover, even if such difficulties could be overcome, there still remains a problem whether we can regard such nodules as the signs of liver cancer development or not. Some of those nodules may not show an autonomous proliferation but show the regressive outcomes, although they might be frequent in the rats sacrificed just after the stop of MC-painting.

One of the interesting results in the present experiment was that the longer the term of DAB-feeding given to rats was, the more intensive and frequent became the formation of metastasis of the developed tumors and the higher their transplantability. This seemed to be analogous to the result of the experiment conducted by Shubik,[7] Salaman-Roe[8] that the more carcinogenic hydrocarbone was given to mice, the more marked the development of malignant skin tumors. However, the intensity of the proliferating ability of a "cancer" is difficult to judge only from histo-pathological findings of an induced tumor, and therefore, the experiment of transplantation is necessary. The relation between the given doses of carcinogenic substances and the autonomous proliferating potency of the induced tumors should be fully taken into consideration, even when the mechanism of azodye carcinogenesis is divided into two phases.

Summary

Five groups of Japanese common albino rats received 0.06% DAB-diet from 1 to 5 months respectively, and, after that, each group was divided into control and

experimental groups. After the stop of the feeding, the rats in the experimental groups were painted twice a week, with a 20-methylcholanthrene solution in acetone, and the treatment continued until the 9th experimental month.

(1) The liver findings of the rats sacrificed immediately after the stop of the feeding, (2) the development of tumors and the histopathological liver change, of the rats which died or sacrificed between the 210th and 420th experimental day, and (3) the transplantability of the induced tumors were examined. The results were as follows:

I) If DAB-feeding was continued until liver nodules which showed the histological picture of adenomatous hyperplasia were produced, adenomas and liver cancers could be developed, even when the animals were fed only the basic diet after the stop of DAB-feeding.

II) If DAB-feeding was stopped before the above nodules were produced, and the animals were left without any treatment, no tumor developed. However, if MC-painting was added after the stop of DAB-feeding, some of the nodules were surely developed to liver cancers. But, in these cases, the cancer formation rate was lower than in the groups which received an additional DAB-feeding.

III) The longer the term of DAB-feeding of a group was, the higher was the transplantability of the induced tumors and the more frequent the rate of metastasis in the original animals. MC-treatment had no influence on these points.

On the basis of the above results, the concept of two-phase mechanism on azodye carcinogenesis, and the relation between the doses of applied carcinogens and the autonomous proliferating potency of induced tumors were discussed.

This work was carried out under the direction of Prof. Tomizo Yoshida, University of Tokyo, and Director of this Institute, to whom the author is greatly indebted for invaluable suggestions and criticism during the course of this investigation.

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Explanation of Plates XIX~XXIV

Fig. 1: Cholangiofibrosis. Distinct adenomatous proliferation of bile ducts and innumerable ductlets. The liver cell cords are separated to irregular, small cell clusters by those proliferated bile ducts (DAB-feeding: 2 mos. Killed at 62 expl. days).

Fig. 2: Intraacinary proliferation of bile ductlets and slight degeneration of liver cells. Acini of the liver are divided into many irregular parts by them (DAB-feeding: 2 mos. Killed at 62 expl. days).

Fig. 3: Cyst formation in the liver. The wall of cavities are composed of a layer of flat or cuboidal epithelia of the proliferated bile ducts (DAB-feeding: 3 mos. Killed at 96 expl. days).

Fig. 4: Adenomatous hyperplasia in the liver. The lesion (upper part) is composed of the cells which have relatively small in size, comparatively enlarged nuclei and slightly basophilic cytoplasms (DAB-feeding: 4 mos. Killed at 134 expl. days).

Fig. 5: Adenomatous hyperplasia (the left half in the field) (DAB-feeding: 4 mos. Killed at 134 expl. days).

Fig. 6: Liver cell adenoma of the liver. Marked proliferation of hepatic cells with distinct nuclei and slightly basophilic cytoplasms. Irregularly shaped clusters of those cells covered with lining cells of the sinusoidal space. (DAB-feeding: 5 mos. Killed at 162 expl. days).

Fig. 7: Liver cancers (Type I). Cord-like arrangemnt of cancer cells. Each cell cords and blood capillaries meet directly or with only an endothelium between them (DAB-feeding: 5 mos. Killed at 277 expl. days).

Fig. 8: Liver cancers (Type II-1). Cord-like and partially adenomatous arrangement of cancer cells including PAS-positive substances in their cytoplasms and cavities (DAB-feeding: 5 mos. Killed at 282 expl. days).

Fig. 9: Liver cancers (Type II-2). Marked adenomatous structures of cancer cells, provided with little connective fibers as stroma (DAB-feeding: 5 mos. Killed at 245 expl. days).

Fig. 10: Metaplastic cartilage formation in the fibrous connective tissues, which was found in the tumors of Type II-2 (DAB-feeding: 5 mos. Killed at 277 expl. days).

Fig. 11: Squamous cell metaplasia of cancer cells which was found in the part of the tumors belonging to Type II-2. (DAB-feeding: 5 mos. Killed at 322 expl. days).

Fig. 12: Liver cancers (Type III). Intensive duct formation of cancer cells and marked proliferation of connective tissues as stroma (DAB-feeding: 5 mos. Killed at 270 expl. days).

Fig. 13: Liver cancers (Type IV). Clusters of cancer cells proliferated irregularly in the fibrous stroma (3 mos. of DAB-feeding followed by 6 mos. of MC-painting. Killed at 348 expl. days).

Fig. 14: Degeneration and necrosis of liver cell cords and marked dilatation of both central

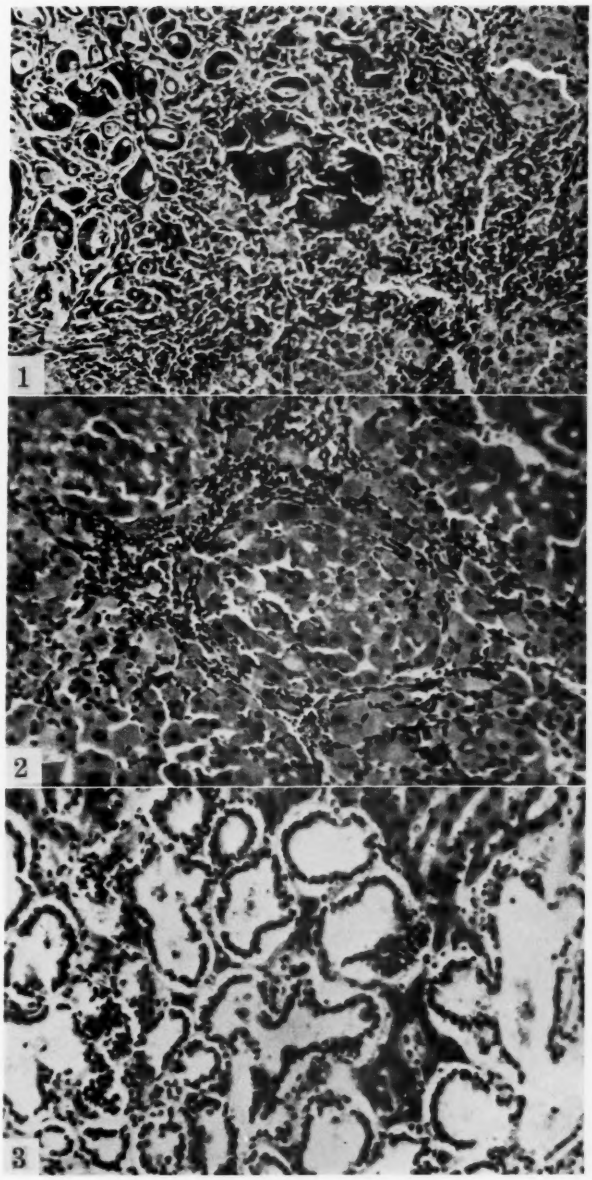
veins and intralobular network of blood capillaries (3 mos. of DAB-feeding followed by 6 mos. of MC-painting. Killed at 420 expl. days).

Fig. 15: Liver adenomas developed in the Group II-C. The adenoma was consisted of several small adenoma nodules separated by a little collagenous fibers (2 mos. of DAB-feeding followed by 7 mos. of MC-painting. Killed at 420 expl. days).

Fig. 16: Higher magnification of the area A in Fig. 15. Liver cell adenoma with arrangement of cells in cord-like fashion. Note the polymorphysm of cells.

Fig. 17: Higher magnification of the area B in Fig. 15. The cell cord, proliferated irregularly forming thick layers with or without endothelium of blood capillaries. Polymorphysm of cells is distinct.

Fig. 18: Cyst formation in the liver. The cells lining the cyst walls proliferated in the cavities (DAB-feeding: 5 mos. Killed at 392 expl. days).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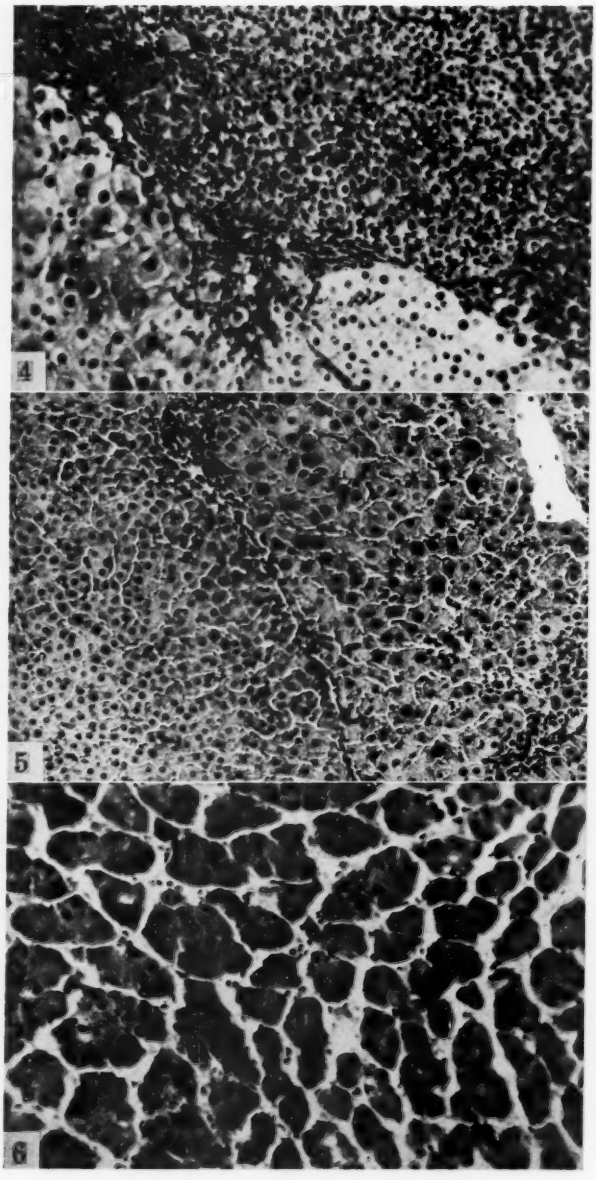
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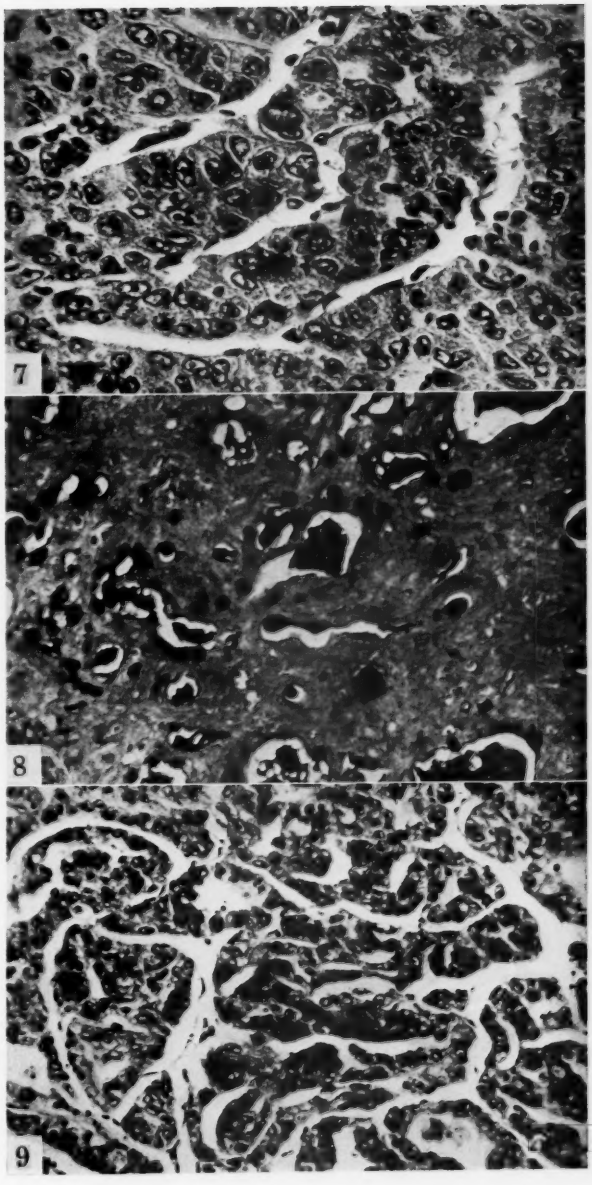
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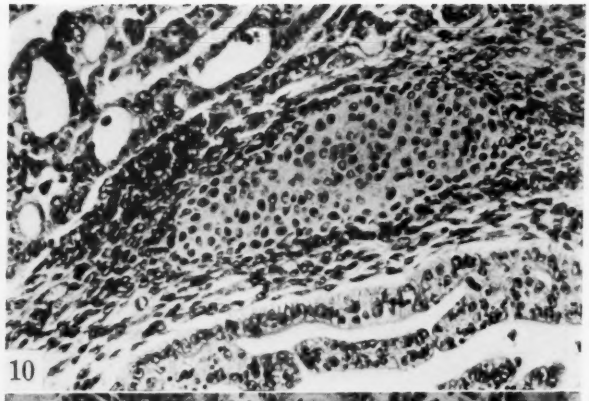

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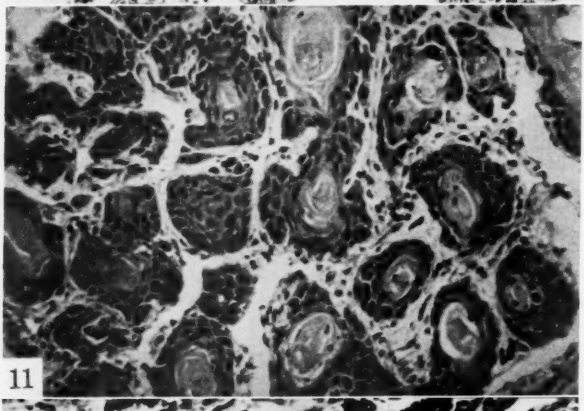

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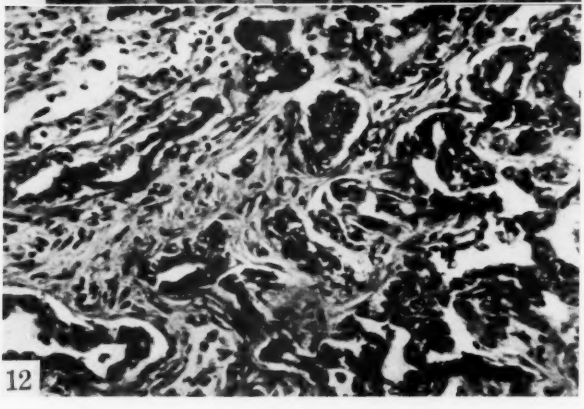

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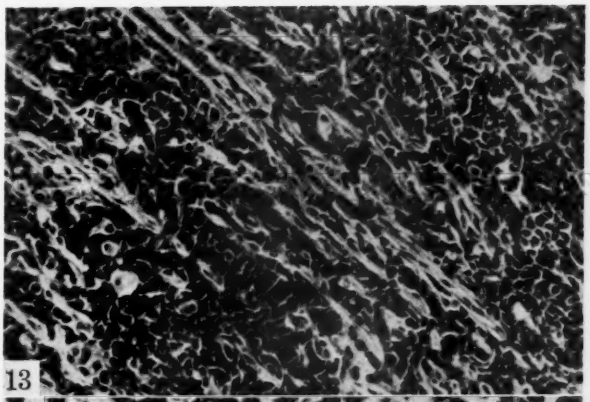
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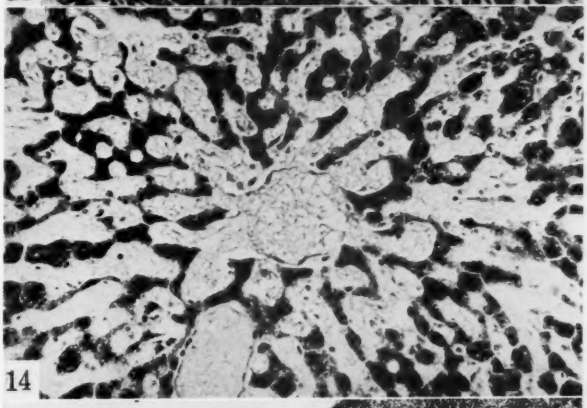
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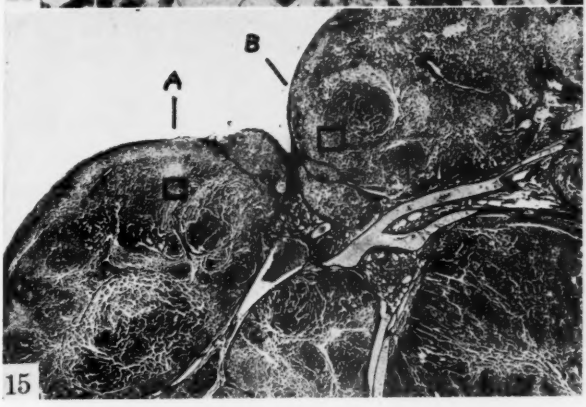

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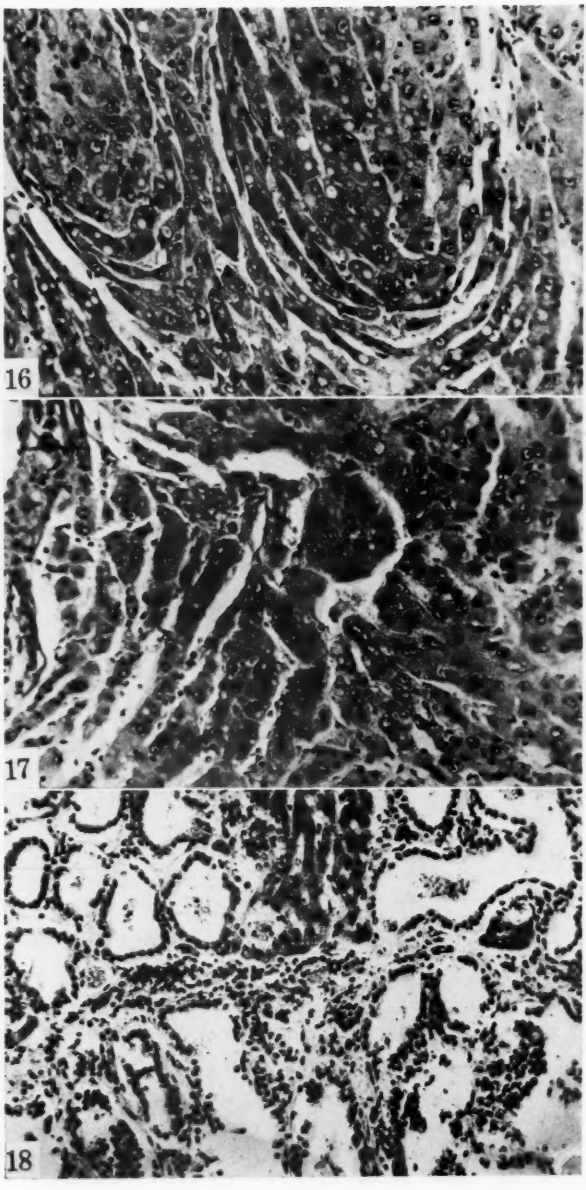
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[GANN, **50**, 347～357 ; December, 1959]

PURIFICATION OF TOXOHORMONE BY DEAE-CELLULOSE COLUMNCHROMATOGRAPHY

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INTRODUCTION

Since Nakahara and Fukuoka (1948)[1),2)] succeeded in extracting a liver catalase depressing factor, toxohormone, from malignant tumors, the efforts for purifing it have been continued in this laboratory. As reported previously by the authors in this journal[3),4)], by the procedure of methanol-acetic acid extration, which had been demonstrated to be useful in extracting adrenocorticotropic hormone (ACTH) from pituitary gland, an active polypeptide fraction free from nucleic acid contamination was obtained from the raw toxohormone fraction prepared from rat Rhodamine fibrosarcoma by the original method of Nakahara and Fukuoka. These fractions were subjected to further purifications by means of cellulose column-chromatography and / or crystalization of active fragment as picrate by the authors[4)].

In the hope of demonstrating the presence of the active substances in the blood stream of tumor-bearing rats, the same procedure was applied for the acetone dried powder of serum protein. But an unexpected production of activity from the inactive serum protein of normal rats was suggested in the course of these experiments[5)], making it necessary to exclude the possibility of artificial production of activity during the boiling methanol-acetic acid extraction of raw toxohormone by using a milder procedure.

In this respect, the method of diethylaminoethyl-cellulose (DEAE-C) column-chromatography may be expected to be most suitable, because it consists of only mild procedures and has been adopted more and more frequently in purifing proteins or enzymes. For example, Sober *et al.* (1956)[6)] employed the DEAE-C chromatography for the separation of serum protein, and succeeded in subdividing the globulin fraction into each component, and to isolate some chromoproteins including methemalbumin. The superiority of this method consists not only in its mild experimental condition, suitable even for the unstable chromoprotein, but also in its excellent resolution of the proteins surpassing all other methods. Moreover the large charging capacity of this ion exchange cellulose is not expected with the other ion exchange resins, and offers great advantage for our experimental purposes.

In the experiments described in this paper, the experimental conditions for the fractionation of the raw toxohormone as well as its methanol-acetic acid extract by the DEAE-C columnchromatography were established, the distribution of toxohormone activity among the resulting fractions was examined, and some properties of the active fractions were pursued.

Material and Methods

Preparation of toxohormone. Toxohormone used in this experiment was the ethanol precipitate of boiling water extract from Rhodamine fibrosarcoma of rat, which was prepared according to the procedure described originally by Nakahara and Fukuoka[2].

Boiling methanolic acetic acid extraction from toxohormone, to obtaine the so called TO-fraction in our laboratory, was carried out as reported previously in this journal[3].

The solutions of samples used for the chromatography were centrifuged after dialysis against distilled water at least for 5 days, and the supernatants were bufferlized to the initial condition of chromatography. Then, an aliquot containing decided amount of protein was applied cautiously on the cellulose exchanger, which has been bufferlized to the same buffer of initial condition of development.

Synthesized anion exchanger, DEAE-Cellulose. The anion exchanger was synthesized from standard grade of Whatman cellulose powder according to the method of Peterson and Sober[7].

This synthesized exchanger was estimated to have the protein adsoption capacity of 60 mg of protein per 100 mg of adsorbent. The protein used in this estimation was bovine plasma albumin (Fraction V of the Armour Laboratories, Chicago, Illinois).

Preparation of column. In Experiment I, DEAE-cellulose used as the exchanger was packed in the column of 1.0×76.0 cm. In Experiments II and III were used the column size of 3.0×8.5 cm. They were washed with several column volumes of 0.005 M Na-phosphate buffer at pH 7.1 and then mounted above an automatic fraction collector (Toyo-Roshi Co., Tokyo). The volume of one collection was adjusted to be 2.0 to 10.0 ml.

Chromatographic procedure. Bufferlized sample concentrated to 2-3 ml was loaded on the column described above and developed by stepwise elution changing buffer composition, pH and ionic strength. Details of each developing solvent system were described in the notes under Figs. 1, 2 and 5. In changing solvent component, the chromatocolumn was washed continuously with at least one column volume of the same buffer until no more elute could be detected in effluents.

All collected tubes were covered and stored in a refrigerator until determination.

And dialysis process was performed also in a refrigerator.

Determination of eluates. In the case of Experiments II and III, the aliquots from collections were diluted 50–100 fold, and examined in a Beckman DK2-type ratio recording spectrophotometer at 210 mμ. Solvent for the dilution used was 0.1 M HCl and for reference blank was used the corresponding dilutes of the same buffer as the test sample. The absorption at 260 mμ was determined at the same time, by which the presence of nucleic acid was estimated.

In Experiment I, the elution curve was established by the reaction of trinitrophenylation. The reaction mixture was composed of 0.5 ml aliquot from each tube, 2.0 ml of 0.2 M phosphate buffer at pH 8.0, and 2.0 ml of 0.1% of TNBS aq. (2, 4, 6–Trinitrobenzene sulfonate), incubated at 40°C, and then the reaction was stopped by the addition of 1.0 ml 6N HCl after 2 hours[8),9)], when the reaction is completed. After dilution with 1.0 N HCl to 20 fold, the reaction products were measured in a Beckman DK 2 spectrophotometer by reading the extinction at 340 mμ, which is the maximal absorption of trinitrophenyl derivatieves. The treatment of trinitrophenylation was carried out in a darkened room.

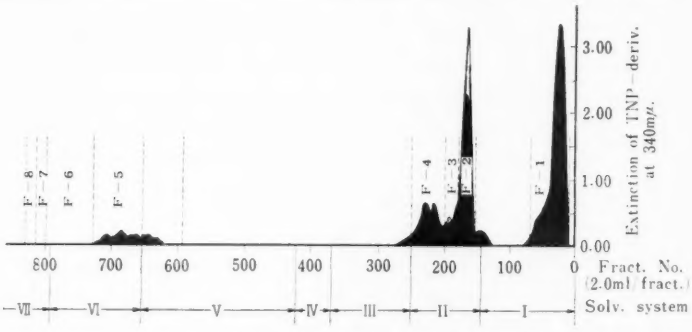
Determination and estimation of each fraction. As shown by elution curve of each figure, the tubes composing each peak were combined in a cellophane bag, then dialysed against distilled water and lyophilized. Lyophilized preparation was dissolved in 10.0 ml of distilled water after weighing. The amount of protein of each fraction was measured by Biuret color reaction and / or calculated directly from the extinction at 210 mμ of chromatographic elution curve. The extinction coefficient value of protein at 210 mμ was calculated as 30.0 from that of 0.1 per cent solution of bovine plasma albumin by our pre-experiment, and this value conformed well to the reference values[10)].

Bioassay of each fraction. Because the amount of original toxohormone charged on the column was expected to be enough to depress the liver catalase activity for 20 normal mice, one-twentieth amount of each fraction was injected intraperioneally per mouse. In one experimental group, 5 mice were used. After 24 hours the injected mice were sacrificed to examine liver catalase activity by von Euler-Josephson's method[11)] with a little modification, which has been used routinly in our laboratory. Ordinarily, experimental results were compared with the appropriate controls, and the mean per cent decrease values calculated. The qualitative judgement of the results was represented as ╫, + and −, depending on the degree of activity. The example of judgement was presented in Fig. 3, that is, ╫ for the sample which lowered the catalase activities in all the tested mice discretely below those of control group, + for that which exhibited distinctive tendency to decrease catalase activity but with a little overlaping with control values, and − in the case in which the range in the experimental group

overlaped completly with that of control.

Results

Fractionations of the raw toxohormone. In Fig. 1 was shown the elution diagram of toxohormone developed stepwisely from the DEAE-cellulose column. The starting buffer, 0.005 M Na-phosphate buffer at pH 7.1, was continued to succesive washing, after which pH was reduced and ionic strength was increased stepwisely as indicated in the notes under the figure. The peaks in the elution diagram



Column size: 1.0×76.0 cm. Solvent system:
I 0.005 M Na-Phosphate buffer (pH 7.1)
II 0.05 M Na-Phosphate buffer (pH 6.5)
III 0.05 M Na-Phosphate buffer (pH 6.0)
IV 0.10 M Na-Phosphate buffer (pH 5.5)
V 0.10 M NaH_2PO_4 (pH 4.7)
VI 0.20 M NaH_2PO_4 (pH 4.7)
VII 0.10 M HCl

Fig. 1. DEAE-C Columnchromatography of Toxohormone (Exp. I)

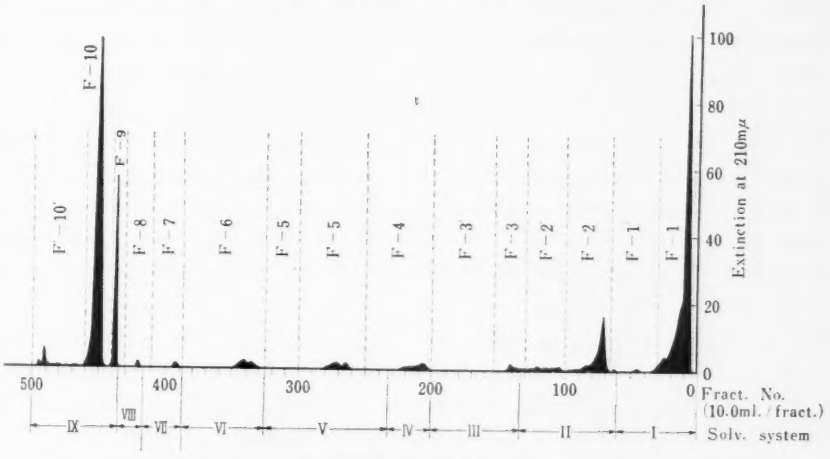
Table 1. The Yield and Catalase Depressing Activity of Each Fraction in Exp. I.

Fraction	Dry weight mg	Yield %	Catalase assay		
			Dosage* mg/head	Result	
				% decrease	Judgement
F-1	172.9	14.4	8.65	45	+
F-2	40.5	3.4	2.02	42	++
F-3	43.3	3.6	2.16	51	++
F-4	10.0	0.8	0.50	46	+
F-5	8.6	0.7	0.43	34	∓
F-6	25.8	2.1	1.29	21	−
F-7	12.4	1.0	0.62	47	+
F-8	5.1	0.4	0.03	0	−

* 1/20 amount of each fraction was injected per mouse.
Total Yield: 318.6 mg (26.6%).
1.2 g of Original Toxohormone was charged on the column.

followed by trinitrophenylation reaction were distributed in 8 fractions, F-1 to F-8, and their yields and activities were summarized in Table 1. Total recovery as protein accounted for only 26.6% of the start, but almost all of the activities were obtained from the fast moving fractions, F-1 to F-4, so that the active principle could be assumed to be eluted with 0.05 M Na-phosphate buffer at pH 6.5 in this chromatographic condition.

Because the recovery of the charged protein was estimated as less than to be expected, and a considerable amount of the loaded material seemed to remain in the column, further elution with an alkaline solution was carried out as shown in Fig. 2 (Experiment II). The elution curves were made up by the absorption of each tube at 210 mμ and by trinitrophenylation procedure as employed in Experi-



(Column size: 3.0×8.5 cm.)

Solvent system:

I 0.005 M Na-Phosphate buffer (pH 7.1)
II 0.05 M Na-Phosphate buffer (pH 6.5)
III 0.05 M Na-Phosphate buffer (pH 6.0)
IV 0.10 M Na-Phosphate buffer (pH 5.5)
V 0.10 M NaH_2PO_4 (pH 4.7)
VI 0.20 M NaH_2PO_4 (pH 4.7)
VII 0.10 M HCl
VIII dist. water
IX 0.10 M NaOH

Fig. 2. DEAE-C Columnchromatography of Toxohormone (Exp. II)

Table 2. The Yield and Catalase Depressing Activity of Each Fraction in Exp. II. (Toxohormone; 1.2 g)

Fraction	Dry weight mg	Yield %	Catalase assay		
			Dosage mg/head	Decrease %	Judgement
F-1	231.5	19.3	11.6	42	+
F-1′	35.7	3.0	1.8	31	−
F-2	68.9	5.7	3.5	43	+
F-10	158.8*	13.2	7.9	52	+

* The value was estimated by extinction at 210 mμ.

ment I. The both chromatographic patterns established by these different methods conformed well to each other, but the former was found to show higher sensitivity and revealed finer peaks than the latter. In the final fraction, F–10, was recovered about 13.2 per cent of protein as estimated by the absorption at 210 mμ, in which, however, was contained also nucleic acid component. The recovery and the activity in catalase depressing action of each main peak in this experiment were summarized in Table 2. It was found here that the greater part of their activities was recovered in the eluates of 0.05 M Na–phosphate buffer, pH 6.5, and also in the final eluate with 0.1 N NaOH.

The chemical natures of the active fractions. Each of the active fractions in Experiment II, F–1, F–1' and F–2, could be divided into two components by the solubility at alkaline pH 10–11, that is, alkaline soluble and insoluble ones. As illustrated in Fig. 3, the active substances were concentrated in the alkaline insoluble component, especially in that from F–2 fraction, which was effective in depressing liver catalase activity with 500 μg dose as protein per mouse.

The ultraviolet absorption curves of these fractions, illustrated in Fig. 4, were obtained by using Beckman DK 2 ratio recording spectrophotometer. It will be seen that the original toxohormone preparation shows two major peaks, one at around 210 mμ of peptide bonds, and the other at 260 mμ for nucleic acid. Upon fractionation these two peaks become separated, the peptide peak in F–2 fraction and nucleic acid peak in F–10.

The amino acid compositions of F–1 and the alkaline insoluble component from F–2 were examined by the two-dimensional paper chromatographic technique, after complete hydrolysis with 6 N HCl for 24 hours at 105°C in oil bath. The

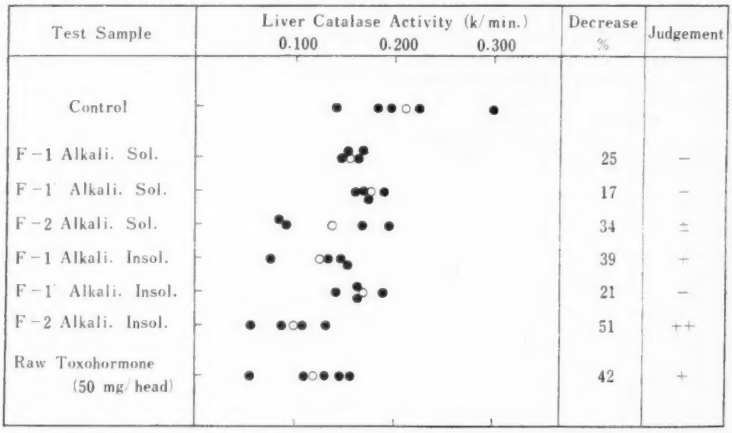
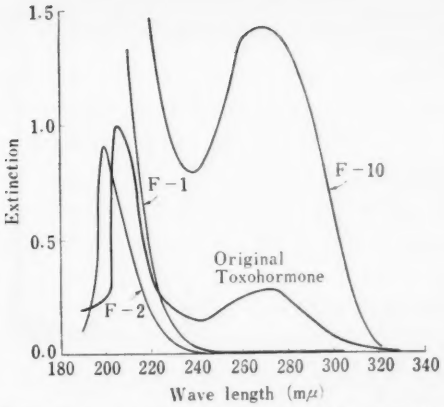


Fig. 3. The Assay of Catalase Depressing Activities of the Alkaline Soluble and Insoluble Portions of F–1, F–1' and F–2 Fractions in Exp. II.



F-1: tube No .5. F-2: tube No. 71. F-10: tube No. 450. = 50 fold dilt. with 0.1 N HCl

Fig. 4. The Ultraviolet Absorption Curves of F-1, F-2, F-10 and Original Toxohormone Fractions in Exp. II.

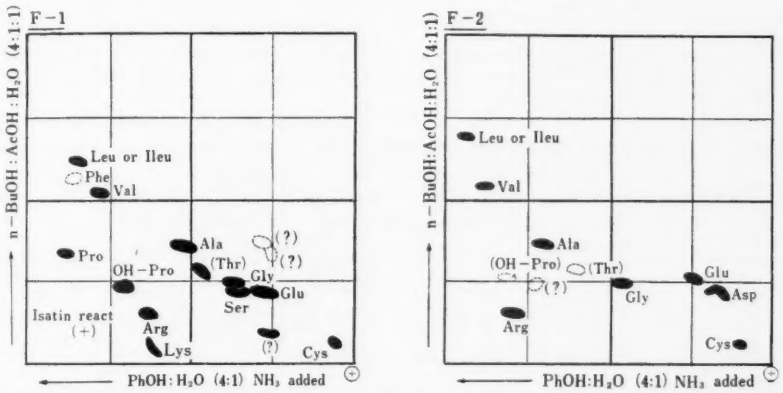
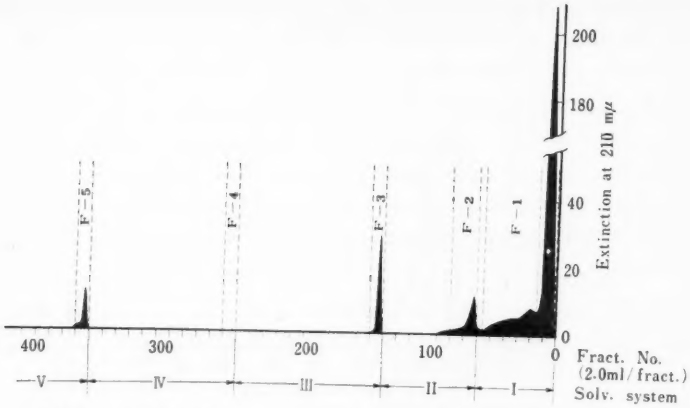


Fig. 5. The maps of amino acid composition of F-1 and F-2 fractions in Exp. II.

chromatographic map of the amino acid composition of each fraction was presented in Fig. 5. It was demonstrated through this qualitative test that about 13 spots of known amino acids including hydroxyproline, which was confirmed by isatin reaction, and 3 unidentified spots were found in F-1. The most active alkaline insoluble fraction of F-2 showed about 10 amino acid spots, including 8 that can be definitely identified, namely, leucine (or isoleucine), valine, alanine, arginine, glycine, glutamic acid and cystine.

Fractionation of TO-fraction by DEAE-cellulose columnchromatography. The methanolic acetic acid extracts of raw toxohormone, TO-fraction, was also subjected to DEAE-cellulose columnchromatography in the same manner as in Experi-



Column size: 1.0 x 5.0cm.
200mg as protein was loaded on the column.

Solvent system:

I 0.005 M Na-Phosphate buffer (pH 7.1)
II 0.05 M Na-Phosphate buffer (pH 6.5)
III 0.10 M HCl
IV dist. water
V 1.0 M NaOH

Fig. 6. DEAE-C Columnchromatography of Methanol-Acetic Acid Extract from Toxohormone (Exp. III)

Table 3. The Yield and Catalase Depressing Activity of Each Fraction in Exp. III. (Methanol-Acetic acid Extract of Toxohormone)

Fraction	Recovery of Protein mg	Yield %	Catalase assay		
			Dosage mg/head	Decrease %	Judgement
F-1	116.5	58.1	2.9	52	++
F-1′	13.0	6.5	0.3	42	+
F-2	4.5	2.3	0.1	57	++
F-3	3.4	1.7	0.1	49	+
F-4	0.2	0.1			
F-5	2.4	1.2			
Total	140.0	70.0*			

* 200 mg as protein was charged on the column.

ments I and II. In this case, however, the elution procedure was carried out only by changing ionic strength of phosphate buffer at fixed pH. In Fig. 6 was shown a chromatogram obtained with TO-fraction. Comparing to the results with the raw toxohormone fraction shown in Experiments I and II, there was observed larger recovery of protein in the fast moving fraction of the elution diagram.

The catalase depressing activity was confirmed in F-1 and F-2 fractions as summarized in Table 3.

Discussion

Due to difference in the column size and the amount of charging sample, the details of the elution patterns showed some differences between Experiments I and II of DEAE-cellulose columnchromatography of toxohormone. But the most active fraction was eluted from the column in the same first two fractions in both cases. For these reasons, the procedure for eluation adopted for this chromatography may be assumed as established for the purpose of obtaining active fraction from toxohormone.

In Experiment III with methanolic acetic acid extract of the raw toxohormone, there came out the active fraction also in the first two peaks of elution pattern as with raw toxohormone, and no more fractions were obtained after those peaks. But the yield of these active fractions exceeded those in Experiments I and II. Although it might not be entirely safe to assume the identity in the nature of these active peaks from toxohormone and its methanolic acetic acid extract only by the position of elution pattern, the results in Experiments I, II and III may be considered to suggest the possibility that the active components originally present in the raw toxohormone are extracted and concentrated in the methanolic acetic acid extract.

As reported previously, the methanolic acetic acid extract of toxohormone is free from nucleic acid, and it gave no fraction by alkaline elution. On the other hand the raw toxohormone fraction contains nucleic acid as much as 30 per cent of its weight, and as demonstrated in Experiment II gave a fraction containing nucleic acid, which was also active as toxohormone. But because this fraction was revealed to contain polypeptide also, it is not sure from this experiment alone which is responsible for the activity of this fraction, protein or nucleic acid.

About the chemical nature of the active fractions in Experiment II, F-1 and F-2, which were active in 11.6 mg and 3.5 mg per mouse, respectively, there was no doubt as to their polypeptide nature from the following reasons: Their ultraviolet absorption curves exhibited only the peaks at around 210 mμ due to peptide bond, lacking those due to nucleic acid. They gave negative reaction in Molish's test. The estimation of Biuret reaction also suggested that these fractions were composed of polypeptide almost entirely.

Although these active fractions were obtained only by one kind of chromatography, and may yet be supposed to contain components other than toxohormone, their amino acid compositions may be important in revealing the nature of these fractions. The paper chromatographic pattern of their amino acid compositions, especially that of the most active F-2, proved to be rather simple, being composed of only 8 main spots. It is an interesting point that there was observed so close

a similarity between the amino acid composition of F-2 and those shown to be necessary by Nakahara and Fukuoka[12] in the biosynthesis of toxohormone *in vitro*. It is also consistent with the early findings of Okushima[13] and the authors[4] who noted the absence of tyrosine in their toxohormone preparations. The presence of hydroxyproline was distinctive in F-1 but was in trace, though not negligible, in F-2. The presence of hydroxyproline in the picrate was the main objection to its homogenity, and so may be also to that of these fractions of DEAE-cellulose columnchromatography.

The fact that the active substances could be eluted out in the fast moving fractions from the DEAE-cellulose column, which is anionic exchanging in nature, might be taken to indicate the cationic state of toxohormone in the neutral reaction. If so, toxohormone may be sure to be retained by cationic exchange column, for example that of carboxymethyl-cellulose. A further purification of toxohormone might be attained by means of that procedure. Also, as demonstrated in Experiment II, the alkaline precipitate from the active fractions retained the major portion of the original activity, but it contained a large amount of inorganic phosphate, suggesting the posibility that the active substances could be adsorbed on the surface of the precipitate of inorganic phosphate. If that is the case, calcium phosphate gel adsorption technique may prove useful in preparing toxohormone without denaturation. The work along these lines will be reported in a subsequent paper.

Summary

The technique of diethylaminoethyl-cellulose (DEAE-cellulose) columnchromatography was employed for the fractionation of the raw toxohormone as well as its methanolic acetic acid extract. Under these experimental conditions, the almost all of the original activity was found in the fast moving fractions, equally in the both cases.

Thus, the possibility was suggested that the active components originally present in the raw toxohormone are extracted and concentrated in the methanolic acetic acid extract. The active fractions were demonstrated to be composed of polypeptide. The most active toxohormone was obtained as alkali insoluble components from F-2 fraction, which was effective in 500 μg dose per mouse as protein, and its amino acid composition was examined by two dimensional paper chromatography after acid hydrolysis.

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PAPER ELECTROPHORETIC STUDIES ON ENZYMES IN THE LIVER OF RATS FED 4-DIMETHYLAMINOAZOBENZENE IV. DESAMINASE OF FATTY ACID AMIDE

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In our laboratory the studies on desaminase activity in the liver of carcinogen fed rats has been carried out by using fatty acid amide and halogenated fatty acid amide as substrates. In continuation of these studies attempts have been made to characterize the enzyme further in detail by introducing the method of paper electrophoresis, as reported in the preceding papers. In the present experiments was examined heat-inactivation of the enzyme in hepatoma and in the normal liver, under the influence of the works of Greenstein and his coworkers on glutaminase and asparaginase, and the results were discussed also from the standpoint of the electrophoresis.

METHODS

Experimental animals and materials.

Male albino rats of our laboratory stock were employed for experiments. The rats were fed with diet containing 4-dimethylaminoazobenzene (DAB) at the level of 0.06 per cent, for the duration of 120 days. After this period DAB feeding was discontinued and the rats were placed on the basal diet without carcinogen for additional 50-60 days. When these rats were autopsied, the livers showed already various grades of lesions. Among the pathologically changed livers, hepatoma nodulus and marked cirrhotic livers were empoyed for the studies. DAB fed rats on their fourth week of experiment have also been included in the study. When the latter rats were autopsied, the livers were normal in gross appearance. Normal male rats fed with basal diet were used for control.

Procedure of the paper electrophoresis.

The rats were sacrificed by cervical dislocation and the livers were removed instantly. The hepatic tissues were homogenized with distilled water exactly three times of their weight in order to make whole homogenates. To obtain supernatants the whole homogenates were centrifuged at 15000 r. p. m. for 1 hour at low temperature.

The fractionation of protein in homogenates have been carried out by introducing paper electrophoretic method in the following manner : the amount of 0.01 ml

of whole homogenate or supernatant was placed on a line on the filter paper strip for every 1 cm in width at the starting boundary, which had been marked across with a pencil, and the current was run for 15 hours under the condition of 400 volts and 2.5 ma. The electrode vessels contained 0.1 M of veronal acetate buffer at pH 8.4. Subsequently, the paper strip was taken off from the apparatus and cut into sections in size of 1×1 cm in case of whole homogenate and 1×3 cm in case of supernatant. Thus 20 segments towards the anode and 2 segments towards the cathode respectively from the starting line were obtained. These segments were used as the enzyme sources.

Determination of the enzyme activity of the paper segments.

Into the outer chamber of the Conway units 1 ml of phosphate buffer at pH 9, 1 ml of 0.1 M solution of substrate (n-caproamide, m. p. 101.5°C or ω-phenylpropionamide, m. p. 102°C), a piece of paper segment and some toluene were transferred successively. Into the central well was brought into 2 ml of 0.02 N H_2SO_4. Then the lids were greased and placed in proper position. After the 18 hours of incubation at 38°C, the distillation of ammonia, which had been liberated from substrate by digestion, was interrupted. Two ml of the Nessler's reagent was added to the H_2SO_4, and the amount of ammonia was measured by a photo-electric colorimeter using the filter of 470 mμ.

The activity pattern of the desaminase of fatty acid amide was then represented graphically for the convenience of the later discussion, namely, in abscissa was taken ammonia nitrogen in γ and in ordinate actual migration range of the enzyme from the starting line on the paper in cm.

Heat-inactivation test of the enzyme on a paper strip.

From a paper strip, on which the whole homogenate of normal liver or hepatoma has been distributed by the electrophoresis, were cut off two parallel strips of the same width of 1 cm. One of which was cut into segments in size of 1×1 cm and were delivered separately into small test tubes in succession, each containing 1 ml of phosphate buffer at pH 9.

The test-tubes were kept in a spacious water bath at 60°C for exactly 10 minutes. Then they were removed and chilled at once in ice-water. The contents of each test-tube were transferred into the outer chamber of the Conway unit with the aid of additional 0.5 ml buffer solution.

The further treatment of the activity measurement of the enzyme and the subsequent completion for making the activity pattern was quite the same as mentioned already. On the other hand the ordinary activity pattern, which had been drawn up from the adjoining intact paper strip, was represented together in Figures 17 and 18.

Heat-inactivation test of the enzyme in whole homogenates.

For the experiment whole homogenates were prepared as follows: in case of normal liver in 100 times of dilution and in case of hepatoma in 20 times of that. The whole homogenates were transferred in about 3–4 ml amounts in each test-tubes separately and they were then dipped into a water bath at the temperature of 50°, 55°, 60°, and 65°C respectively for 10 minutes. After that the test-tubes were taken out from the water bath and immersed immediately in ice-water for cooling. The measurements of the desaminase activity were then carried out by using the Conway units in the above whole homogenates, both of normal liver and hepatoma, compared with original intact whole homogenates respectively. Into the outer chamber of the units were pipetted 1 ml of 0.1 M veronal–HCl buffer at pH 9, 1 ml of 0.1 M n-caproamide, 1 ml of the homogenate regarding as the enzyme source and few drops of toluene. In the central well was kept 2 ml of 0.02 N H_2SO_4 and the units were put with greased lids in proper position and incubated at 38°C for 3 hours. After that the H_2SO_4 was nesslerized and the amount of ammonia was measured in the same way as already described.

The data were represented in Table 1, also graphically by bars in Figure 19, in taking a relative strength of activities to those of intact homogenates both normal liver and hepatoma equally as 100.

Results and Discussion

According to the activity patterns of desaminase of fatty acid amide in whole homogenates of normal liver, cirrhotic liver and the liver of DAB rats on their fourth week of feeding, showed a quite smilar type of pattern not related to the used substrates, n-caproamide and ω-phenylpropionamide (Figs. 1, 2, 3, 5, 6, 7, 17 (a)).

A curve posessing a prominent peak was observed just on the starting line and its low foot remained on the slow moving part of the protein zone. The author interpreted this curve as consisting of two curves as seen in Figure 17 (a) and the bigger one on the starting line overlaped the low one at the slow moving part, which will be discussed later in connection with supernatants. Furthermore a very low convex curve was found in the electromigrated part, where the protein zone began diminishing in its amount.

Whole homogenate of hepatoma, however, showed a trace of activity curve from the starting line to the slow moving part and a very low convex curve was found in the electromigrated zone (Figs. 4, 8, 18 (a).

The activity patterns obtained from the supernatants of the hepatic tissues and hepatoma showed very similar type, namely, two low convex curves were observed, one of which situated at the slow moving part of protein zone. The author thought that the low curve at the slow moving part came out to a certainty by means of

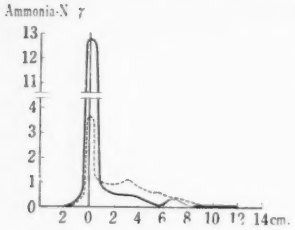

Fig. 1 Normal liver
(Whole homogenate)
Substrate: n-Caproamide

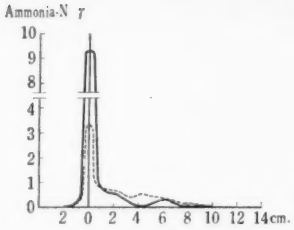

Fig. 2 Cirrhotic liver
(Whole homogenate)
Substrate: n-Caproamide

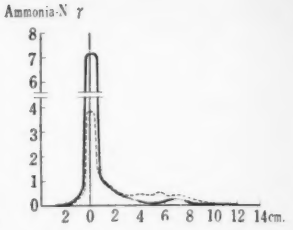

Fig. 3 Liver of DAB rat
(4th week) (Whole homogenate)
Substrate: n-Caproamide

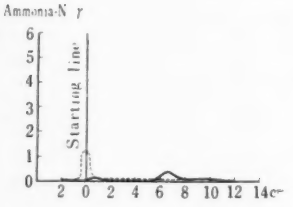

Fig. 4 Hepatoma
(Whole homogenate)
Substrate: n-Caproamide

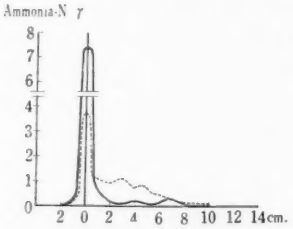

Fig. 5 Normal liver
(Whole homogenate)
Substrate: Phenylpropionamide

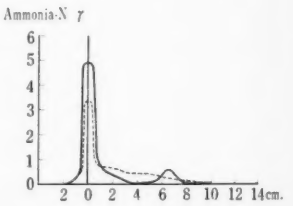

Fig. 6 Cirrhotic liver
(Whole homogenate)
Substrate: Phenylpropionamide

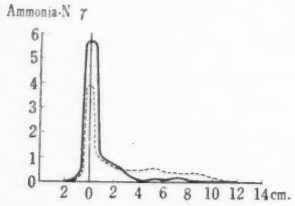

Fig. 7 Liver of DAB rat
(4th week) (Whole homogenate)
Substrate: Phenylpropionamide

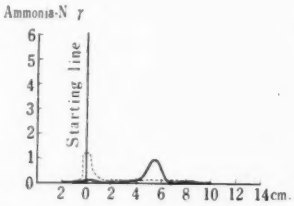

Fig. 8 Hepatoma
(Whole homogenate)
Substrate: Phenylpropionamide

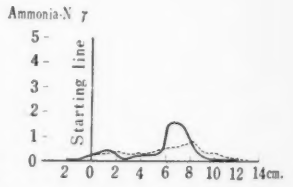

Fig. 9 Normal liver
(Supernatant)
Substrate: n-Caproamide

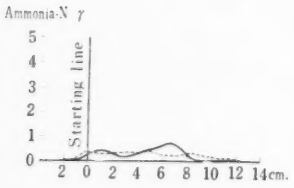

Fig. 10 Cirrhotic liver
(Supernatant)
Substrate: n-Caproamide

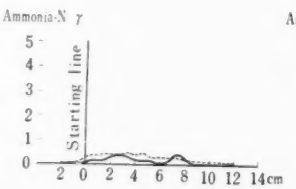

Fig. 11 Liver of DAB rat
(4th week) (Supernatant)
Substrate: n-Caproamide

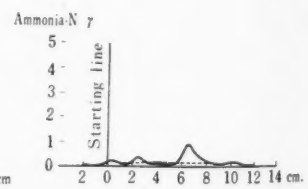

Fig. 12 Hepatoma
(Supernatant)
Substrate: n-Caproamide

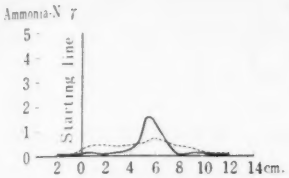

Fig. 13 Normal liver
(Supernatant)
Substrate: Phenylpropionamide

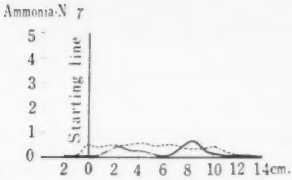

Fig. 14 Cirrhotic liver
(Supernatant)
Substrate: Phenylpropionamide

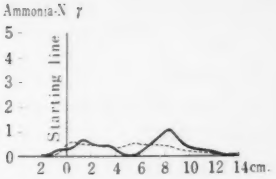

Fig. 15 Liver of DAB rat
(4 th week) (Supernatant)
Substrate: Phenylpropionamide

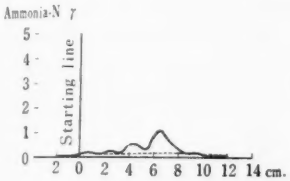

Fig. 16 Hepatoma
(Supernatant)
Substrate: Phenylpropionamide

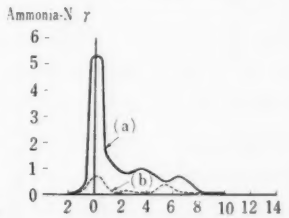

Fig. 17 Normal liver
(Whole homogenate)
Substrate: n-Caproamide

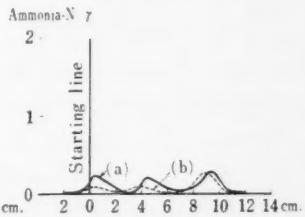

Fig. 18 Hepatoma
(Whole homogenate)
Substrate: n-Caproamide

Figs. 1–16. Figures show the activity pattern of the desaminase (solid curve) of fatty acid amide in the normal liver and the liver of rats fed DAB, accompanied by the distribution pattern of hepatic tissue protein in arbitrary units (light broken curve). The longitudinal line of each figure represents the starting line, onto which tissue homogenate has been applied for paper electrophoresis. The right side of the starting line is towards the anode.

Figs. 17–18. Figures show the activity patterns of the desaminase of fatty acid amide in the normal liver and hepatoma as compared with before and after heat-treatment of whole homogenate of respective tissue. (a) Untreated homogenates. (b) Heat-treated homogenates at 60°C fo 10 minutes.

centrifugation of cell fragments. The other convex curve appeared at the migrated part, and its site was the same as described in the above lines (Figs. 9–16).

The heat-inactivation studies have been carried out by comparing the two activity patterns simultaneously. One was obtained from a paper strip in the usual manner as already mentioned, and the other one from the adjoining strip after treatment with heat.

The pattern of the normal liver altered markedly after heat-treatment, namely, the prominent peak at the starting line was almost lost, whereas the curve at the migrated part remained roughly unchanged (Fig. 17 (a), (b)). The pattern of hepatoma was persistent even after heat-treatment (Fig. 18 (a), (b)).

The heat-inactivation test of the enzyme was extended also to whole homogenates of normal liver and that of hepatoma. The activities of both intact homogenates were taken as 100 and when they have been treated with heat, for instance, at 65°C for 10 minutes, the enzyme activities fell to 2.9 in normal liver and 28.7 in hep-

Table 1

Heat-inactivation studies on the desaminases (substrate: *n*-caproamide) in whole homogenates of the normal liver and hepatoma. The enzyme activities, which are represented by the amount of ammonia-N, of both untreated homogenates are taken as 100.

		Normal liver	Hepatoma
Untreated		100	100
Heat-teatment for 10 minutes	50°C	88.8	97.8
	55°	69.0	88.0
	60°	26.9	56.5
	65°	2.9	28.7

atoma respectively (Table 1, Fig. 19). Therefore the enzyme in hepatoma was just 10 times more heat-stable than that of normal liver under such condition.

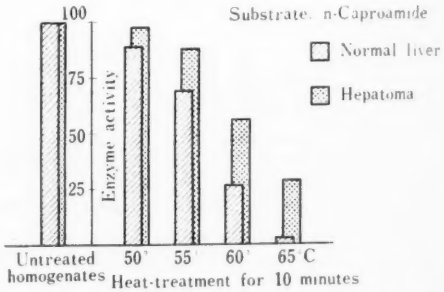

Fig. 19 Heat-inactivation studies on the desaminase in whole homogenates of the normal liver and hepatoma. The data in Table 1 are represented graphically for the purpose of visualizing.

From the above resultus the author suggests, that there are at least two sorts of desaminases of fatty acid amide in the liver of rats, namely, heat-labile and heat stable ones. The author postulates to call the former as desaminase I and the latter as desaminase II. Thus desaminase I in the whole homogenate of normal liver is situated mainly at the starting line and predominated the enzyme activity. Desaminase II is located in the fast moving part and the intensity of its activity was far less than that of desaminase I. The small enzyme activity in hepatoma was due to desaminase II and thus the enzyme in hepatoma was considered to be heat-stable.

Sammary

1) The desaminase activity of fatty acid amide in the hepatic tissue homogenate of rats fed with 4-dimethylaminoazobenzene was discussed according to the patterns of enzyme activity, which has been obtained by the method of paper electrophoresis. Moreover the heat-inactivation of the enzyme has been examined.

2) Whole homogenates of hepatic tissues except hepatoma showed an activity pattern with a sharp peak on the starting line and a low convex curve at the fast moving part of protein zone. In case of hepatoma the marked peak on the starting line of the pattern was almost imperceptible. In contrast to this all supernat-

ants of hepatic tissues including hepatoma showed two low curves in the patterns, one at the slow moving part and the other at the fast moving part.

3) Heat-inactivation test to the enzyme on the paper strip has been measured. Whole homogenate of the normal liver lost the prominent peak on the starting line of the pattern, whereas that of the hepatoma the pattern remained almost unchanged even after heat-treatment.

4) Heat-inactivation test of the enzyme was extended to whole homogenates of both normal liver and hepatoma. The enzyme in hepatoma was far more heat-stable than that of normal liver.

5) The author postulates to classify the enzyme at least in two sorts, i. e., heat-unstable desaminase I and heat-stable desaminase II. The former predominated in the activity of the whole homogenate of normal liver and the latter was represented in the whole homogenate of hepatoma.

This investigation was supported in part by a grant-in-aid from the Japanese Ministry of Education (S. K.).

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Correction to Asano's previous paper (Gann, Vol. 49, 287-294, 1958). For "Ammonia-N γ" in Figs. 1-13, read "p-Nitrophenol γ".

[GANN, **50**, 367～373; December, 1959]

STUDIES ON THE AMINE OXIDASE IN THE LIVER AND OTHER TISSUES OF RATS FED WITH 4-DIMETHYLAMINOAZOBENZENE

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Three years ago the author with co-workers published some studies on the monoamine oxidase activity in the liver of rats fed with hepatic carcinogens, 4-dimethylaminoazobenzene (DAB) and 2-acetylaminofluorene, using n-butylamine, n-amylamine and tyramine as substrates.

The author has now extended the investigation to diamine oxidase, employing histamine and cadaverine as substrates. The tissues examined were not only the liver, but also kidneys, pancreas, brain, heart, muscle, spleen, adrenals, testes and lungs of rats fed with DAB.

In this study tyramine oxidase has also been included as one of the monoamine oxidases in tissues, in ordes to compare with the experiments on diamine oxidase.

Materials and Methods

Experimental animals. Albino rats of male sex of our laboratory stock were maintained on the diet containing 0.06 per cent of DAB for about 150 days, then on normal diet for more than 30 additional days. On autopsy of these rats noticeable changes were found macroscopically in the livers of majority of animals. Then the rats were classified according to the extent of their liver lesions as follows: (I) rats with cirrhotic liver and (II) hepatoma-bearing rats.

Rats fed with DAB diet in their fourth week of experiment (III) were also examined; the livers of these animals showed usually normal appearance.

Furthermore the following animals were employed, i. e., in untreated rats were performed the partial hepatectomy, with the excision of about 40 per cent by weight of the liver and the rats were sacrificed on 48th hours following the surgery. These rats were introduced as (IV) partially hepatectomized rats. The rats which had been merely laparotomized and were sacrificed on 48th hours postoperatively were called (V) sham-operated rats.

For control were used the intact rats of similar weight, which had been fed normal diet.

Enzyme sources. The following organs were excised from the above mentioned

animals, namely, (1) the liver, (2) kidneys, (3) pancreas, (4) brain, (5) heart, (6) muscle (quadriceps femoris), (7) spleen, (8) adrenals, (9) testes, and (10) lungs. Especially, the livers of rats of long-term DAB application were subdivided according to the appearance, into three as follows: the liver of uneven surface, cirrhotic liver and hepatoma nodulus. They were homogenized with distilled water exactly 50 times of the weight of tissue and used as the source of the enzyme.

Determination of amine oxidase activity. Conway units were employed for the determination. Into the outer chamber of units were delivered 1 ml of 0.1 M tyramine, histamine or cadaverine respectively (the latter two were originally dihydrochlolide and were neutralized by NaOH prior to use) and 2 ml of 0.1 M phosphate buffer at pH 8 with a few drops of toluene. Three ml of 0.02 N H_2SO_4 were transferred into the central compartment of the units. Then the lids were vaseline-greased and fixed in proper position and the units were incubated at 38°C for 20 hours. At the end of the time digestion was interrupted by opening the units and 2 ml of Nessler's reagent was pipetted into the H_2SO_4 and the amount of absorbed ammonia was measured by a photo-electric colorimeter by using the filter 470 mμ. Accordingly the activity of the enzyme was represented by the amount of liberated ammonia-N in γ. The mean values of several measurements obtained from 4-8 individuals were indicated graphically by bars in the figures.

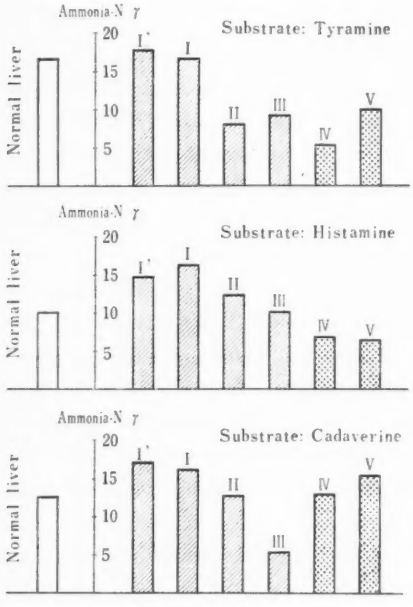

Fig. 1. LIVER

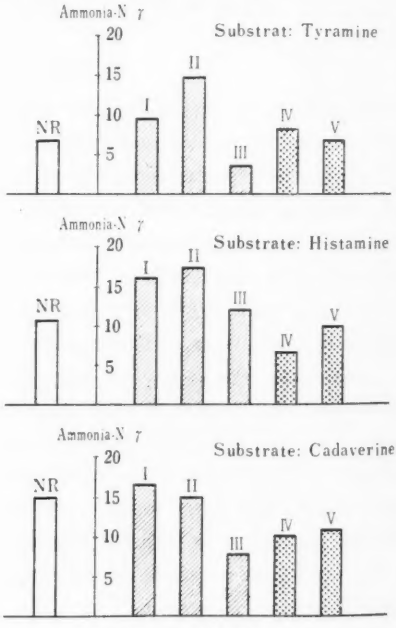

Fig. 2. KIDNEYS

Results and Discussion

Amine oxidase activity of several organs and tissues in rats fed with DAB has been measured. Among the organs the liver was discussed as a separate item because it is the major site of tumor formation (Fig. 1).

In Fig. 1, unshaded bars represent the activity of amine oxidase in the normal rat-liver in terms of ammonia-N in γ.

Shaded bars show the activity in hepatic tissues of rats fed with DAB, i. e., the liver of uneven surface (I'), cirrhotic liver (I), heptoma nodulus (II) and the liver of rats in their 4 th week of experimet (III).

Dotted bars show the activity in the regenerating liver (IV), and in the liver of sham-operated rats (V).

Tyramine oxidase activity of hepatoma diminished about to the half of the normal level and that of the liver of uneven surface and cirrhosis had a height not less than the normal level, as had been described in the previous paper.

The activities of histamine and cadaverine oxidase in hepatoma nodulus showed the similar or rather increased activities as compared with those of normal liver. This was a remarkable difference between monoamine and diamine oxidases concerning with hepatoma.

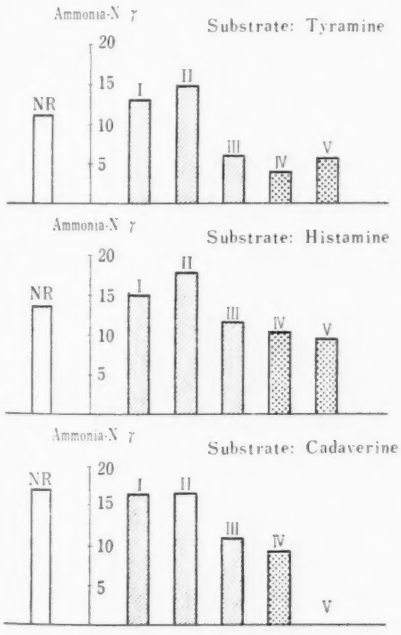

Fig. 3. PANCREAS

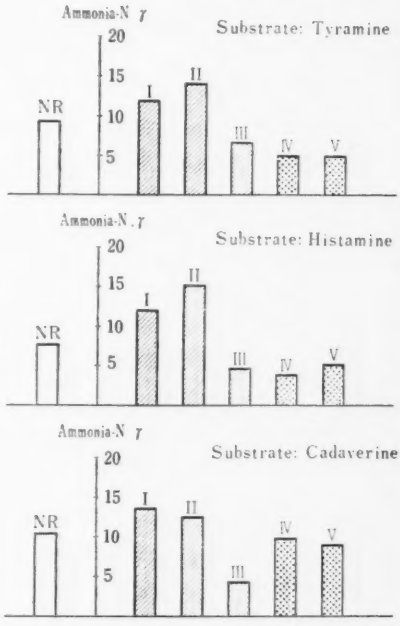

Fig. 4. BRAIN

Diamine oxidase activities in the pathological but noncancerous liver were increased compared to that of the normal liver and the result was more manifest than in case of tyramine oxidase.

The livers of DAB fed rats in their early experimental days showed low levels of tyramine and cadaverine oxidase activities. Accordingly the fed dye may have an effect on depletion of tyramine and cadaverine oxidase in the liver. In this case the diminution of histamine oxidase activity was not distinct.

For comparison the liver of partially hepatectomized and sham-operated rats have been similary tested. The regenerating liver was less active in tyramine and histamine oxidase than the normal liver. When cadaverine was used as substrate, however, the above differences were not demonstrable.

The liver of sham-operated rats showed amine oxidase to be less active than the normal liver when tested by means of tyramine and histamine, but in case of cadaverine oxidase the above difference was not recognizable.

The activities of tyramine, histamine and cadaverine oxidase in the kidneys, pancreas, brain, muscle, spleen, adrenals, testes, and lungs of DAB fed rats have also been measured (Figs. 2–10). The above rats were in the course of hepatocarcinogenesis and the organs treated were distant from the site of precancerous or

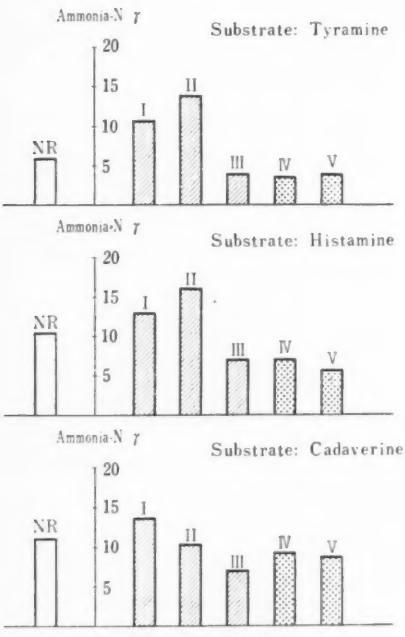

Fig. .5 HEART

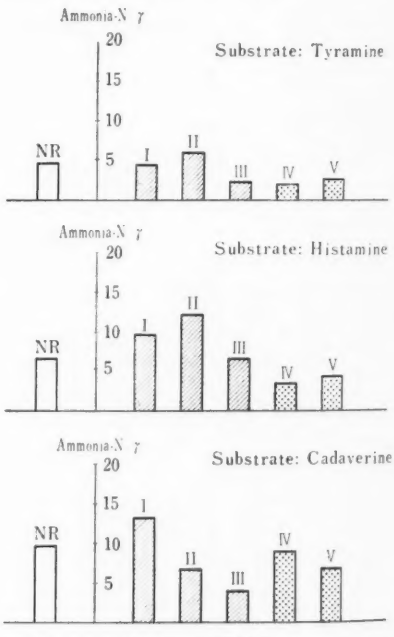

Fig. 6. MUSCLE

cancerous tissue, i. e., hepatic tissue.

In Figs. 2–10, unshaded bars show the activity (indicated by ammonia-N in γ) of amine oxidase in several tissus, namely, kidneys (Fig. 2), pancreas (Fig. 3), brain (Fig. 4), heart (Fig. 5), muscle (Fig. 6), spleen (Fig. 7), adrenals (Fig. 8), testes (Fig. 9), and lungs (Fig. 10) of normal rats (NR).

Shaded bars represnt the activity of the enzyme in the above tissues of rats fed with DAB, i. e., rats with cirrhotic liver (I), hepatoma-bearing rats (II), and rats in their 4th week of experiment (III).

Dotted bars show the activity of the enzyme in the above tissues of partially hepatectomized rats (IV) and sham-operated animals (V).

The organs and tissues of hepatoma-bearing rats had higher level of activities of monoamine and diamine oxidase than the corresponding tissues in normal rats, when tyramine and histamine have been employed as substrates. The most remarkable example was the adrenals in case of histamine oxidase test (Fig. 8).

When cadaverine was treated the activity difference of the enzyme between normal tissue and tissue of the tumorous hosts was usually of similar height.

The organs and tissues in rats with cirrhotic liver showed the activity of the enzyme not less than or higher than those of control rats. Noteworthy was the fact that the correlation between the cirrhotic liver and the host was becoming

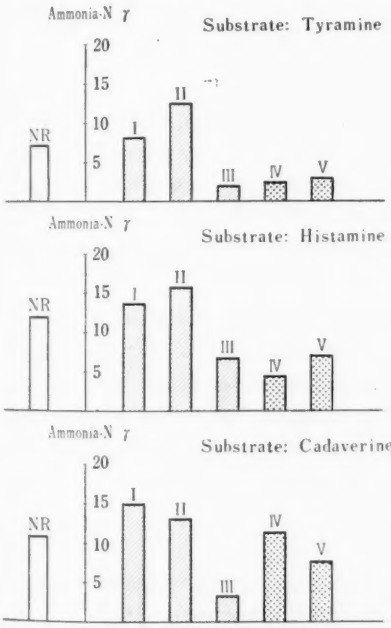

Fig. 7. SPLEEN

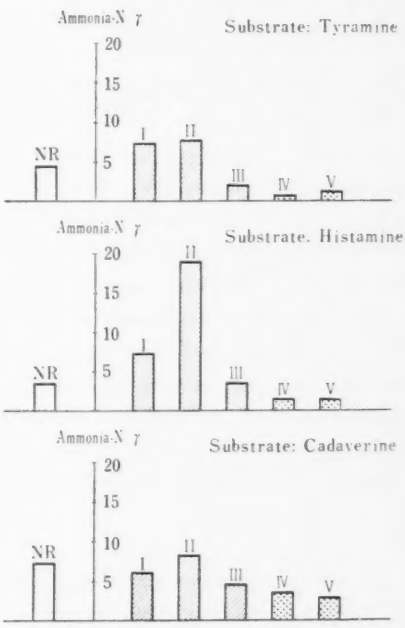

Fig. 8. ADRENALS

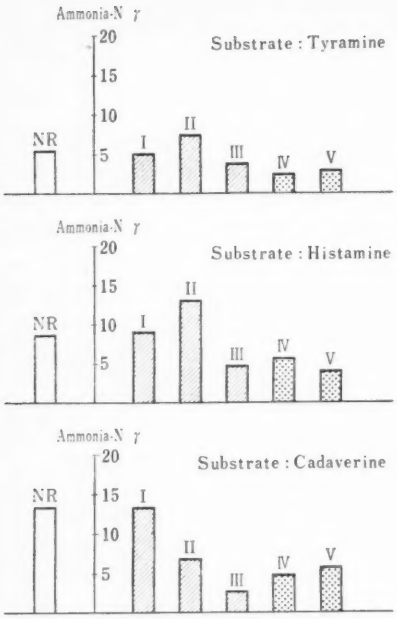

Fig. 9. TESTES

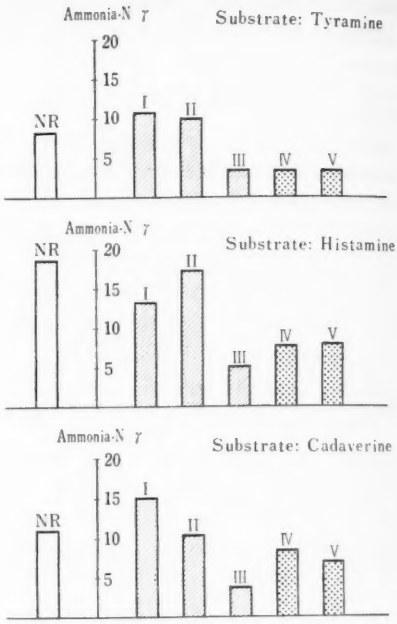

Fig. 10. LUNGS

akin to that of hepatoma and the tumorous host.

The inhibiting effect of fed DAB on the amine oxidase activity is considered, as it shows low levels in the organs and tissues of DAB fed rats already in their fourth week of experiment. So the enzyme depleting action of administered DAB might have taken place in every part of the body.

The organs and tissues of partially hepatectomized and sham-operated rats showed lower activities than those of normal rats.

In conclusion a discussion will be made once more from the general point of view. Hepatoma and the regenerating liver were originally at a high rate of mitosis, nevertheless they had different effects on the distant organs, i. e., hepatoma induced in them an increased activity of amine oxidase, while the regenerating liver produced the contrary effect. From these facts the author considers that the tumor is not simply a tissue with a rapid growth rate.

Sammary

1) Using histamine and cadaverine as substrates, the activity of diamine oxidase in the liver and the other organs of 4-dimethylaminoazobenzene (DAB) fed rats have been measured as compared to those of corresponding organs of normal rats. The activity of tyramine oxidase was also examined as an example of monoamine

oxidase. The similar studies have also been carried out concerning the partially hepatectomized and sham-operated rats.

2) The activity of diamine oxidase in hepatoma was similar to that of normal liver. The activity of the enzyme in pathological but noncancerous liver were increased over that of normal liver. Whereas the activity of tyramine oxidase in hepatoma was remarkably low, that in the liver of uneven surface and cirrhotic liver did not so differ from that of normal liver.

3) The rats with cirrhotic liver had a high level of mono- and diamine oxidase activity in the kidneys, pancreas, brain, heart, spleen, etc. The organs of hepatoma-bearing animals showed usually noteworthy height of activity, conspicuous example being the adrenals, when histamine oxidase was tested.

4) The liver and other organs of DAB rats in their early experimental days showed low mono- and diamine oxidase activity compared with those of normal rats.

5) The liver and other organs of partially hepatectomized and sham-operated rats showed the similar tendency of the enzyme activity to that described in the above lines (4).

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INITIAL STAGES OF GLYCOLYSIS IN TESTIS AND MALIGNANT TISSUES

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An analytical method for the ion exchange separation and determination of sugar phosphates including fructose-1-phosphate (F-1-P) was presented previously (1). This method was applied on several tissues to obtain more exact informations about the initial stages of glycolysis. Several data on liver, muscle and brain were already published elsewhere (2). This communication will offer some informations on the early stages of sugar utilization in the rapidly proliferating tissues, i.e., testis and malignant tissues.

Materials and Methods

Materials:

Glucose, glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), fructose, fructose-6-phosphate (F-6-P), and fructose-1, 6-diphosphate (FDP) used were the commercial products. F-1-P was prepared from FDP by using rabbit bone phosphatase (3).

As a rapidly proliferating normal tissue, rabbit testis was used. Yoshida ascites tumor cells and human mammary carcinoma were also examined.

Reaction mixture:

Four ml of 33 per cent isotonic KCl homogenate of normal and malignant tissues was mixed with 1 ml of 0.056 M fructose and other substrates, 1 ml of 0.1 M ATPNa, 1 ml of 0.05 M $MgCl_2$, and 1 ml of 0.5 M NaF. The mixture was adjusted to pH 7.0 with 0.38 M $KHCO_3$, and the final volume was made up to 10 ml. Aliquot of the above mixture was analysed at once without incubation, and another same mixture was incubated at 37°C in a constant temperature bath.

Analytical Methods:

The reaction mixtures, before or after incubation anaerobically, was deproteinized by the addition of 10 ml of 10 per cent perchloric acid and neutralised with 5N KOH. The neutralised solution was deionized by means of Dowex 50 (acid form), and then adjusted to pH 8.5 with dilute aqueous ammonia. Sugar phosphates in the deionized solution were analysed on Dowex 1 column (0.9 $cm^2 \times 10.5$ cm) by the method described previously (1).

Results

(1) Rabbit testis:

When glucose was incubated anaerobically with testis homogenate, the rapid transformation of glucose to FDP was observed as shown in Table 1. Some amount of

Table 1. Incubation of glucose.

	Glucose	Fructose	G-1-P	G-6-P	FDP
Initial (μM)	23.2	0.43	0	0.2	0.32
3 min. (μM)	21.4	0.22	0.25	0.6	1.65
7 min. (μM)	19.0	0.48	0	2.87	3.10

Table 2. Incubation of glucose-1-phosphate.

	G-1-P	Glucose	G-6-P	FDP	Unidentified sugar phosphate, perhaps cyclic glucose phosphate *
Initial (μM)	15.6	1.17	1.85	0	12.6
5 min. (μM)	0	3.95	3.80	2.74	3.24

* as glucose

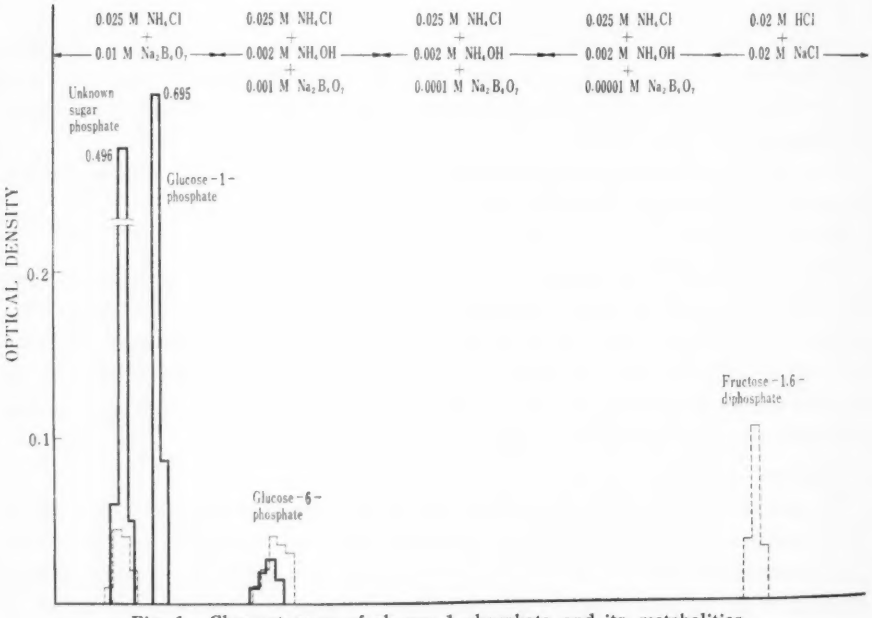

Fig. 1. Chromatogram of glucose-1-phosphate and its metabolities incubated with rabbit testis homogenate for 5 minutes.
—— initial, - - - - after 5 minutes

G-1-P was always found in the early time of incubation.

When G-1-P was incubated with testis homogenate, 100 per cent of this sugar phosphate added disappeared after 5 minutes incubation, and the increase of glucose, G-6-P and FDP were quite small compared with G-1-P disappearance (Table 2). An unknown compound which moves faster than G-1-P was often detected in some old samples of G-1-P (Fig. 1). Although the structure of this unknown compound has not been examined in detail, it may be doubly esterified glucose phosphate, because this compound was formed by alkali treatment of the fresh samples of G-1-P which showed just one peak at first. Some old samples of F-1-P also contain an unknown compound as shown later in Fig. 2, but the latter moves a little faster than the former contained in the G-1-P sample.

When G-6-P was incubated, about 70 per cent of G-6-P disappeared within 5 minutes incubation, and glucose and FDP were the predominant products (Table 3).

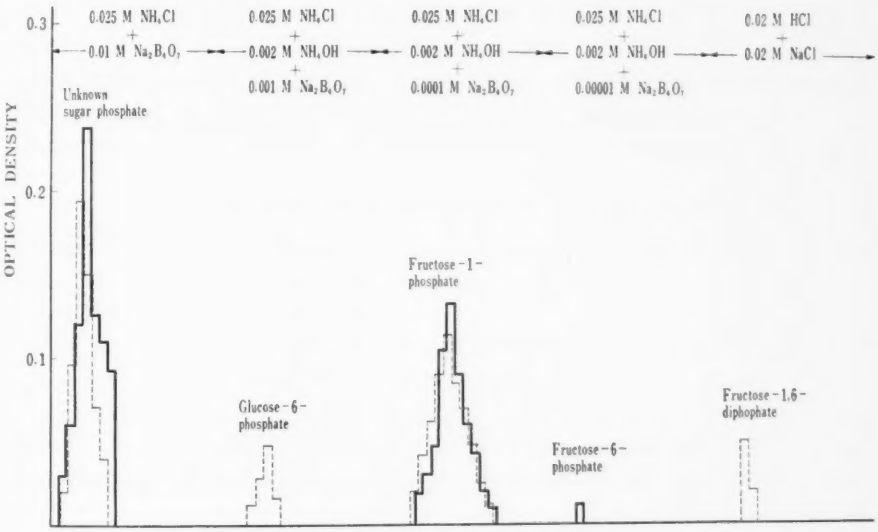

Fig. 2. Chromatogram of fructose-1-phosphate and its metabolites incubated with rabbit testis homogenate for 5 minutes.
—— initial - - - - after 5 minutes

Table 3. Incubation of Glucose-6-phosphate.

	G-6-P	F-1-P	F-6-P	FDP	Glucose	Fructose
Initial (μM)	10.08	0.52	0.35	0.35	0.99	0.31
5 min. (μM)	3.11	0.17	0.35	1.70	2.03	0.53

Akaeda (4) has reported that the glucokinase of rabbit testis is C-1-glucokinase by measuring the acid soluble phosphate before and after incubation. But the decision of testis glucokinase whether it phosphorylates C-1 or C-6 position of glucose may be still too early. The fate of rapidly disappeared G-1-P should be chased in the future.

When fructose was incubated with the testis homogenate, no fructose monophosphates were detected, and G-6-P and FDP were the predominant products as shown in Table 4.

Table 4. Incubation of fructose-6-phosphate without added ATP.

	Fruction	Glucose	G-1-P	G-6-P	FDP
Initial (μM)	19.08	—	—	—	—
3 min. (μM)	14.8	0.9	0.61	1.69	0.64
5 min. (μM)	12.4	0.7	0.5	2.15	2.12
10 min. (μM)	10.39	0.6	0	4.3	3.0
20 min. (μM)	9.05	2.61	0	3.91	2.9

When F-6-P was incubated without added ATP, amazingly rapid conversion of F-6-P to G-6-P was observed, and G-6-P produced was gradually dephosphorylated to free glucose (Table 5).

Table 5. Incubation of fructose-6-phosphate without added ATP.

	F-6-P	Fruction	Glucose	G-1-P	G-6-P	FDP
Initial (μM)	16.43	0.42	0.45	—	0.85	0.19
5 min. (μM)	1.72	—	1.72	0.16	14.35	0.17
10 min. (μM)	1.78	0.18	4.33	0.42	10.3	—
20 min. (μM)	1.76	1.31	7.25	0.40	4.95	—

When F-6-P was incubated with ATP, rapid transformation of F-6-P to G-6-P was also observed, but increment of glucose was small and FDP production was increased (Table 6).

Table 6. Incubation of fructose-6-phosphate with added ATP.

	F-6-P	Fructose	Glucose	G-1-P	G-6-P	FDP
Initial (μM)	14.5	—	—	—	—	—
1 min. (μM)	0	0.89	0.87	0.69	9.78	1.73
5 min. (μM)	0	1.11	0.70	0.46	10.86	2.14

The fate of added F-1-P was shown in Fig. 2 and Table 7. In Fig. 2, an unknown peak was also seen before the peak of G-1-P. This unkown compound, which is

Table 7. Incubation of fructose-1-phosphate.

	F-1-P	Glucose	G-6-P	F-6-P	FDP	Unidentified sugar phosphate, perhaps cyclic fructose phosphate *
Initial (μM)	6.53	0.91	0	0.15	0	20.6
5 min. (μM)	6.77	3.18	2.02	0	0.79	13.6

* as fructose

often detected in some old samples of F-1-P but not in the fresh samples, may be doubly esterified sugar phosphate, because this compound has the following characteristics: a) general reaction for sugar (phenol-H_2SO_4 method (5)) was positive; b) reducing reaction for aldose (6) was negative; c) ketose reaction (cysteine-carbazol-H_2SO_4 method (7)) was negative; d) Fiske Subbarow's reaction for inorganic phosphate (8) was negative; e) on paper-chromatogram (9), the organic phosphate reaction was positive; f) after hydrolysis, ketose reaction and Fiske Subbarow's reaction were positive but aldose reaction was negative. From Table 7, it may be reasonable to consider that the observed increment of glucose and G-6-P after incubation of F-1-P may be due to the transformation of this unknown sugar phosphate to those sugars, and F-1-P itself is not metabolized by testis.

Thus the fructokinase of testis is considered to be C-6-fructokinase as pointed out by Akaeda (4).

Two unknown compounds which are present in the old samples of G-1-P or F-1-P, appear to be metabolized by testis. However, it is not clear at present how and why these compounds are metabolized in the testis.

(2) Yoshida ascites tumor cells:

When glucose was incubated with Yoshida ascites tumor cells anaerobically, FDP was the predominant product and the small amount of G-6-P was produced. But no fructose-monophosphates was detected at any time of incubation (Table 8). These results may indicate that the transformation of glucose to FDP via G-6-P is quite rapid in these malignant cells.

Table 8. Incubation of glucose with Yoshida ascites tumor cells.

	Glucose	Fructose	G-6-P	FDP
Initial (μM)	25.0	0.1	0	0.12
5 min. (μM)	20.6	0.1	0.7	3.66
10 min. (μM)	17.6	0.1	1.02	6.10
20 min. (μM)	9.2	0.15	1.49	13.6

When fructose was incubated, again no fructose monophosphates was detected at any time of incubation, and FDP was the predominant product (Table 9).

Table 9. Incubation of fructose with Yoshida ascites tumor cells.

		Fructose	G-6-P	FDP
Initial	(μM)	25.4	—	—
10 min.	(μM)	21.8	0.4	2.7
20 min.	(μM)	19.6	0.68	4.5

Table 10. Incubation of fructose-6-phosphate with Yoshida ascites tumor cells.

	F-6-P	Fructose	Glucose	G-6-P	G-1-P	FDP
Initial (μM)	21.0	0.18	0.82	1.63	0	0.25
10 min. (μM)	0	0.25	1.86	5.44	0.2	16.3

Table 11. Incubation of fructose-1-phosphate with Yoshida ascites tumor cells.

	F-1-P	Glucose	Fructose	G-6-P	FDP	Unidentified sugar phosphate, perhaps cyclic fructose phosphate *
Initial (μM)	14.95	1.4	0.15	0.96	0	39.8
10 min.(μM)	14.55	0.15	4.21	1.41	2.37	24.8

* as fructose

The fate of F-6-P and F-1-P incubated were in the same situation as in the case of testis (Table 10, 11).

So the fructokinase of these malignant cells may have C-6-fructokinase.

(3) Human mammary carcinoma:

Only fructose was tested in this case. As shown in Table 12, this carcinoma tissue may contain C-6-fructokinase.

Table 12. Incubation of fructose with human mammary carcinoma.

	Fructose	Glucose	F-6-P	G-6-P	FDP
Initial (μM)	18.74	0.1	—	—	—
10 min. (μM)	15.5	0	0.26	0.48	2.04
20 min. (μM)	12.4	0	1.54	1.4	2.38
30 min. (μM)	11.1	0.1	0.63	2.95	2.6

Discussion

When fructose was incubated with the homogenates of liver and muscle, F-1-P production was clearly demonstrated by the author's method, and F-6-P formation was also observed in the case of brain (2).

In the case of testis, however, quite puzzling data were obtained. It can not be decided at present whether testis glucokinase is C-1- or C-6-glucokinase.

Fructokinase of testis may be regarded as C-6-fructokinase, because the rate of disappearance of added F-6-P was comparable with the rate of fructose removal and F-1-P added was not metabolized. Akaeda (4) has shown that the glucokinase of rabbit testis is inhibited by alloxan treatment, but its fructokinase is not affected. So the testis may contain a specific C-6-fructokinase. It is well known that brain hexokinase phosphorylates fructose to F-6-P, but it is not well defined whether brain fructokinase is a different enzyme from its glucokinase or a non-specific enzyme of yeast type. Brain hexokinase is not inhibited by alloxan treatment, so it looks to be an yeast type. But Haugaard *et al.* (10) has stated that insulin can not enter into the brain tissue through the blood-brain barrier, i.e., the brain hexokinase is not under control of insulin. So, at present, it is still obscure whether brain tissue contains two specific glucokinase and fructokinase or non-specific hexokinase of yeast type. From these considerations, it will be infered that the testis fructokinase is the only specific C-6-fructokinase ever found in animal tissues.

Yoshida ascites tumor cells appear to contain C-6-glucokinase, and also C-6-fructokinase.

Human mammary carcinoma appears to have C-6-fructokinase.

But whether the hexokinases of malignant tissues are specific or non-specific enzymes remaines to be determined. Even if the problem discussed above may be solved in the near future, next question may arise why liver and muscle contain C-1-fructokinase and why testis, brain, and malignant tissues contain C-6-fructokinase. It should be added that the rate of each step of glycolysis is quite different from tissue to tissue.

Summary

(1) An analytical method for the separation and the determination of sugar phosphates developed by the author was applied on testis and malignant tissues in order to get more exact informations of the early stages of sugar utilization.

(2) It is still obscure whether testis glucokinase phosphorylates C-1 or C-6 position of glucose, because of the rapid formation and rapid disappearance of G-1-P in this tissue homogenate. The testis appears to have specific C-6-fructokinase.

(3) Yoshida ascites tumor cells may contain C-6-glucokinase and C-6-fructokinase, and their fructose utilization was quite similar in behavior to that of the testis.

(4) Human mammary carcinoma contains C-6-fructokinase.

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[GANN, 50, 383~394; December, 1959]

CYTOLOGICAL STUDIES OF TUMORS. XXIX. MORPHOLOGICAL AND CYTOLOGICAL CHANGES IN THE ASCITES HEPATOMA II OF BUFFALO RATS AFTER TRANSPLANTATION INTO F_1 HYBRIDS, WISTAR-KING A × ALBANY[1)]

(Plates XXV and XXVI)

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The general cytological features and the stemline ideogram of an ascites hepatoma produced in a Buffalo rat by administration of p-dimethylaminoazobenzene were reported in one of the previous papers of this series (Tanaka 1959). The most interesting feature of this tumor is a strong affinity of tumor cells to form masses: the cells after division remain adhered to the original mass without being free from the latter. Serial transfers of this tumor have been carried out in Buffalo rats in an island form for a period of 59 generations without any change in morphological characteristics and chromosomal constitution. However, after serial passages of this tumor with F_1 hybrids between Wistar-King A and Albany rats, the tumor cells in hepatoma-cell islands gradually lost their cellular adhesiveness, and a new cell population consisting of free tumor cells was established in the peritoneal fluid of the rats. By the intraperitoneal inoculation of the ascites fluid containing free tumor cells into new hosts, a free-cell subline has been maintained through serial transfer passages, showing several advantages for cytological researches. Recently, Sato, Belkin and Essner (1956) have witnessed in a C3H mouse ascites hepatoma a similar type of transformation. The sequence seems to be related to either selection or genetical change of tumor cells. It is of considerable interest to inquire into the development of the free-cell subline from the cytological point of view. The present paper deals with a study of morphological and cytological changes in the course of transformation of the ascites hepatoma II into the free-cell subline, with special concern to the genetical change of tumor cells.

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Kyoko Kanô for her valuable advices and criticism. To Dr. Motomichi Sasaki and Miss Akiko Sato, the author is also greatly indebted for valuable technical help and the supply of animals.

Material and Methods: The original ascites hepatoma II and its derivative (free-cell subline) furnished material for the following experiments. The animals used for transfer were Buffalo, Wistar-King A (WKA)×Albany(AL) F_1 hybrid, Wistar, Albany, Long-Evans and Fischer, all being kept in this laboratory. Animals of both sexes at four months of age were used for the experiments.

Routine transplantation of the ascites tumors and withdrawal of the tumor ascites were made with the use of sterilized glass pipettes.

Sections of solid tumors fixed with Orth's fluid were stained with haematoxyline and eosin. The ascites tumors were studied cytologically with acetic orcein or acetic dahlia squash preparations. For observations of the chromosomes, tumor cells were pretreated with distilled water for about 18 minutes prior to the application of acetic orcein or propionic suden black B solution. Succinic dehydrogenase activity of tumors was examined according to Hirono's method (1957).

Results

I. Derivation of the free-cell subline

The ascites hepatoma II (AH-II) is characterized by irregular shaped hepatoma-cell islands. On the 5th day after transplantation, the smallest unit of hepatoma-cell islands consists of two tumor cells, whereas the large island shows 30–40 or some more cells (Fig. 1). The tumor cells mostly measured about 15 to 25 microns in diameter, though giant cells measuring 50 to 60 microns in diameter with one, two or more nuclei were observed. Neoplastic exudate of the tumor-bearing animals also contains a small number of free tumor cell populations below two per cent. Such features had remained without any changes for 59 transfer passages into Buffalo rats from which the primary tumor was originated (for details see Tanaka 1959).

Since there was a difficulty in securing an adequate supply of pure Buffalo rats, F_1 hybrids (WKA×AL) and Buffalo rats were used for experiment. Chart 1 shows the data on transplant history in these rats. The WKA×AL rats in the 60th to 66th

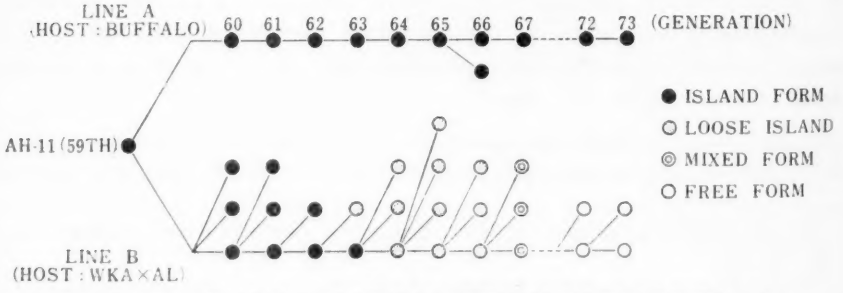

Chart 1. Transplant history of AH-II into WKA×AL hybrid and Buffalo rats.

transfer generations did not show 100 per cent "takes" of the tumor. Noticeable is the fact that the tumor cells forming certain hepatoma-cell islands showed a tendency to separate from each other. Further, the test of the succinic dehydrogenase activity indicated that in some islands, diformazan granules precipitate throughout all areas of the island, while in some others the concentration of diformazan was markedly less (Fig. 2). In general, the islands showing the greater enzyme activity were considerably greater in respect to contiguity of hepatoma cells than those possessing less activity. The meaning of this evidence is unknown, though it may be related to some contiguity effect of hepatoma cells.

In the 67th transfer generation, a change was observed in the hepatoma inoculated in all F_1 rats of WKA×AL: an increase in number of free cells occurred with a concomitant decrease in that of hepatoma-cell islands until they disappear almost entirely from the ascites. The majority of free cells displayed a characteristic amoeboid movement (Fig. 3). The cells elongated in form, having an area of clear ectoplasm forming an undulating membrane in both ends of cells. Occasionally, amoeboid or migrating cells contained granules of varying sizes.

The neoplastic exudate was then inoculated into three WKA×AL rats. Abdominal fluid taken from those rats three days after inoculation was hemorrhagic, and contained numerous free tumor cells together with a few hepatoma-cell islands. Movement of cytoplasmic protrusions of free tumor cells slowed down after inoculation into new rats. On the surface of free tumor cells, knob-like processes of various lengths were protruded, the tip of the protrusions being stained with acetic dahlia or acetic orcein. They decreased in number with the passage of time and finally all tumor cells showed round shape (Fig. 4). It is thus evident that the hepatomas have developed as a free cell population in F_1 rats of WKA×AL. This line has been maintained in hybrid rats for over 80 transfer generations (March 1959) without shift in cytological features being established as a distinct subline which will be referred to as AHF. In striking contrast, the hepatomas growing in pure Buffalo rats have remained in an island form without any change in morphological or chromosomal characters.

II. General properties of the subline

Under experimental conditions, multiplication of the tumor cells of this new subline (AHF) in the peritoneal cavities of hybrid rats proceeded in regular manner. The general characteristics of AHF will be described below in comparison with those of the original AH-II.

Morphological features: Morphologically the tumor cell differs between the original AH-II and subline AHF. The AHF is of free or discrete cell-population, whereas the tumor cells of AH-II have formed characteristic islands. Figure 4 shows the typical appearance of the fluid of AHF stained with acetic dahlia: most tumor cells are 20 to 30 microns in diameter, some being over 60 microns. In general, the average cell

size was somewhat larger than that of the original AH-II. The nucleus was oval or kidney-shaped. Cells containing a ring nucleus were regularly present: they measured 25 to 50 microns in diameter. Large round or rod-shaped necleoli are common. Multinucleated cells were also present.

In the Sudan black B preparations, most tumor cells showed numerous lipid droplets which were distributed throughout the cytoplasm.

When tested with neotetrazolium, the localization of oxidizing system which reduces tetrazolium to formazan was demonstrated in mitochondria, as shown in Figure 5. The cells of small diameter showed rather greater succinic dehydrogenase activity though this correlation was not always distinct, since there occurred occasionally some moderately large cells showing considerable activity, and small ones with low activity.

The behaviour of tumor cells was observed with the aid of a phase contrast microscope in living condition: some of the cytoplasmic and nuclear changes above mentioned were observed rather clearly in living cells.

From the results of the above observations, and based on the comparison with the Yoshida sarcoma and MTK-sarcomas, it is evident that the general feature of the AHF is of a typical nature as an ascites tumor.

Behaviour of the tumor cells in the peritoneal cavity: After intraperitoneal inoculation of tumor cells, there is generally a rapid increase in volume of peritoneal fluid in the body cavity of the host. Within 4 to 6 days after inoculation, the tumor cells reached a state of nearly pure culture, forming milky ascites fluid in the peritoneal cavity of the host. The volume of the fluid increased with the passage of time together with the increase in number of tumor cells. By the 7th to 8th day after inoculation, the peritoneal fluid changed from light pink to deep red in coloration became of a blood content. The death of tumor-bearing animals generally occurred between the 9th and 12th days after inoculation.

Transplantability of the subline into different stock rats: The rate of "takes" following the intraperitoneal transplantation of the AHF into rats of several different stocks is shown in Table 1. The results of the former study of the original AH-II (Tanaka 1959) are recorded in this table for comparison. It is evident from the table that AHF was highly sensitive to rats of various stocks. The tumor cells proliferated

Table 1. Pooled results of transplantation tests in original AH-II and subline AHF.

TUMOR	HOST					
	BUFFALO	WISTAR/Ma	LONG-EVANS	ALBANY	FISCHER	WKA × AL
AH-II	112/112 (100%)	88/104 (84.6%)	25/32 (76.5%)	14/21 (66.6%)	0/20 (0%)	/
AHF	18/18 (100%)	69/74 (93.2%)	20/20 (100%)	18/20 (90%)	11/14 (71.4%)	70/72 (97.4%)

rapidly in rats of the following six stocks, Buffalo, WKA×AL, Wistar, Long-Evans, Albany and Fischer, whereas some stocks were less or non-susceptible to the original AH-II. Interesting it is that transplantability for Fischer rats is remarkably dissimilar between the two tumor lines. No animals died of the original AH-II tumor in Fischer stock. On the contrary, the AHF killed 11 animals out of 14 Fischer rats (71.4 per cent).

The mean survival time of the host was found to be shorter in AHF than in AH-II. For example, transplantation into Buffalo rats resulted in the finding that the survival time was 18 days for original AH-II and 10 to 12 days for AHF. This trend should be considered as an indication of increased viability or virulence of the AHF tumor.

Histological differences between the original tumor and the subline: It is evident from the forgoing observations that the AHF shows typical ascites nature and its virulent malignancy highly raised following alterations of cellular characteristics. In view of the importance of investigation of the histological changes in tumors, a comparative histological examination was done in sections of the original AH-II and the subline, AHF, which was converted into solid form after subcutaneous inoculation.

In histological pattern, the original AH-II, was found to be a typical hepatoma as shown in Figure 6. In general microscopic examinations, the primary tumor and the AH-II showed no visible difference between them (Tanaka 1959), while some histological changes have occuarred in the AHF (Figs. 7 and 8). In the tumor tissue of AHF, the lesions are composed of cells showing a variation in size and shape with general characteristics closely resembling those of the tumor tissue found in other ascites tumors such as MTK-II and Yoshida sarcomas. Round, ovoidal or polygonal cells, measuring from 10 to 30 microns in diamter, are packed closely together to form a homogenous infiltrating tissue. The cell boundaries are distinct. The cytoplasm is non-granular and generally basophilic. The nucleus was relatively large in size and surrounded by a distinct nuclear membrane. It should be mentioned that the sarcoma-like architecture was observed in some portion of the tissue. Such a histological feature has never been observed in the original AH-II.

III. Comparative analysis of chromosomes between the original tumor and the subline

In connection with the histological differences, a comparison of the chromosomes was made between the AH-II and AHF.

Difference in chromosome number: In order to secure comparative data of chromosomes, tumor samples were obtained on the 3rd, 4th and 5th day after inoculation in each tumor. Figures 25 and 26 show the distribution of chromosome numbers in tumors studied. It was observed that the AH-II has 47 as its stemline number, while in AHF the stemline number lies in 38 (Figs. 9-14). It thus became

Figs. 15–24. Ideogram analysis of original AH–II and subline AHF. 15–17 and 21–22, original AH–II tumor. 18–20 and 23–24, subline AHF tumor.

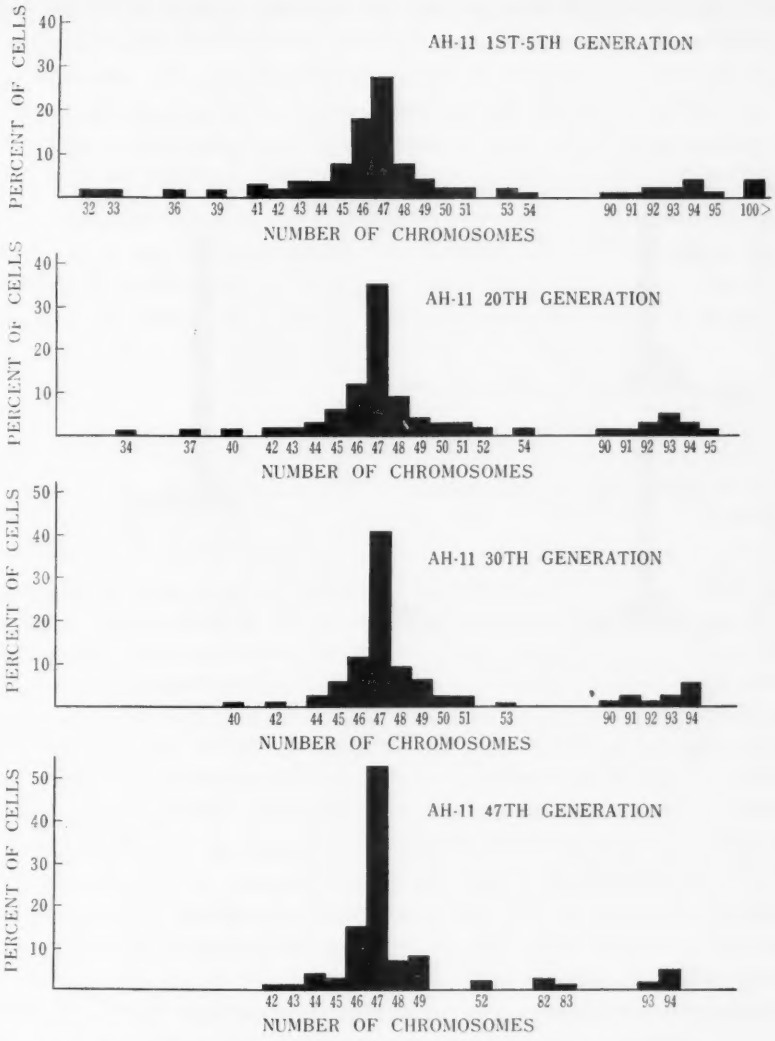

Fig. 25 Graphs showing changes in chromosome number distribution in original AH-II during successive transfer generations.

evident that a definite numerical difference occurs between AH-II and AHF in the stemline cells.

In early transfer generations, cells with the typical stemline number were not observed in the AH-II. In samples from the 1st-5th to 20th generation, there occurred two types of chromosome number, one varying from 32 to 54 and the other

showing from 90 to 104. With the increase in number of generations, the variation range of chromosome number became narrow. The variation range was 40 to 53 for the 30th generation and 42 to 52 for the 47th generation (Fig. 25). The cells showing the stemline number in the 47th generation were 52.5 per cent in occurrence.

In striking contrast, the modal number of AHF was established in early transfer generations (Fig. 26). In the 4th generation the tumor contained the cells with the

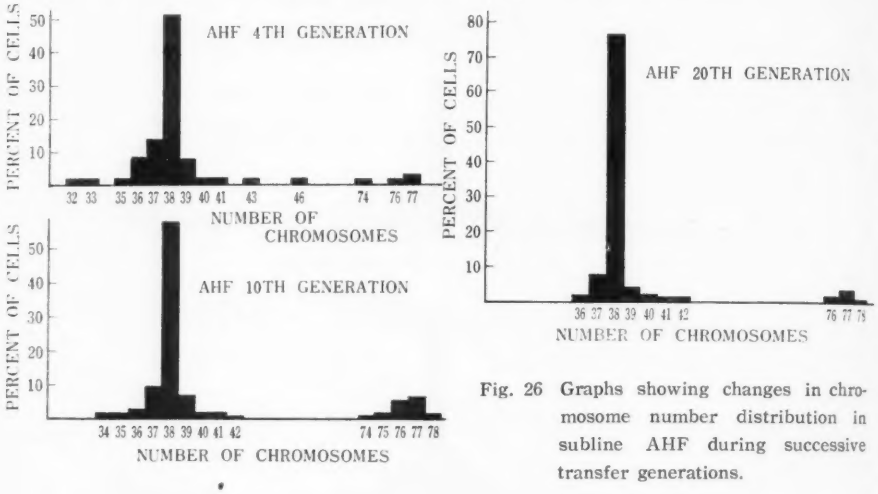

Fig. 26 Graphs showing changes in chromosome number distribution in subline AHF during successive transfer generations.

stemline number at 54 per cent. By the 20th generation, the cells with typical stemline number predominated in occurrence showing over 75 per cent, and the variation around the stemline number was considerably reduced. This condition has continued for 80 subsequent transfer passages. It seems to indicate that the subline AHF is quite stable at the time of development.

Difference in chromosome morphology: The morphological changes of chromosomes were studied in samples taken during the passages of the original AH-II through WKA×AL hybrid rats.

Characteristic chromosome pictures of the two tumor lines, AH-II and AHF, are presented in Figures 9 to 14. By close investigation, it was shown that AHF is characterized by the presence of the following marker chromosomes which grossly distinguish this line from the original AH-II: 1) one largest metacentric chromosome, 2) four large acrocentric chromosomes. Such marker chromosomes have never been observed in cells of AH-II either in the present or past samples studied. The stemline ideograms from nine random samples of AH-II and from ten samples of AHF were analyzed. The chromosomes were classified by comparing their characteristic shape and size, and arranged in three groups as rod-, J- and V-shaped

elements. Three ideograms from each line are shown in Figures 15 to 24 to clarify the difference between AH-II and AHF. For convenience of comparison, the stemline ideograms of the two tumor lines are formulated as follows:

Original tumor, AH-II: 27 (R's)+6 (J's)+14 (V's)=47 chromosomes

Subline tumor, AHF: 14 (R's)+11 (J's)+13 (V's)=38 chromosomes

Further, it is a noticeable fact that larger chromosomes of the AHF consist of J-shaped elements, while in AH-II they are of rod-type. The minute chromosomes always exist in the typical plates of both tumors. They are of about half the size the smallest chromosome. The fact that their occurrence in AHF and AH-II is remarkably constant seems to indicate that they may contain genes of some importance.

Discussion

The results of the present study clearly indicate that the subline tumor, AHF, derived from the AH-II differs cytologically and morphologically from the original tumor. The loss of cellular adhesiveness and the particular stemline ideogram are the most significant characteristics of this subline. On the derivation of this subline there are at least the following theoritical possibilities by which a tumor of new properties might have been produced in WKA×AL hybrid rats: 1) the free cell-population was of natural occurrence, and its growth was suppressed under certain unfavourable circumstances, or 2) certain cells forming a hepatoma-cell island (or islands) underwent mutational changes which led to the loss of cellular adhesiveness.

As described in one of the previous papers (Tanaka 1959), the neoplastic exudate of the original AH-II consisted of a large number of hepatoma-cell islands, but isolated single tumor cells were present in the tumor ascites, though they were very few in number, being less than two per cent. It is supposed that these freely floating tumor cells have undergone selection to form a population through proliferation under certain physiological conditions in the body cavity of hybrid rats from WKA×AL. If this possibility is reasonable, the stem cells of the newly established free cell-population should possess the same chromosomal constitution as the stem cells of the AH-II. Observations resulted in finding that no such cells have been observed in the original AH-II. Further, it seems impossible that the free cells, small in number, would persist alive in the AH-II for 59 transfer generations covering about 6 months.

Further, the possibility of selection is to be decreased on the basis of the results of the transplant experiment of a single free cell obtained from an ascites hepatoma (Yoshida 1956), or of single free cell-population produced by enzymatic digestion of hepatoma-cell islands (Essner, Sato and Belkin 1955, Tanaka 1959). When the single cell or single-cell population was inoculated intraperitoneally, cell division was started,

but daughter cells did not separate and as a result small-cell clumps were produced. Such small cell clumps may be regarded as clones derived from a single tumor cell. It is, therefore, highly probable that the AH-II is not mixed tumor consisting of tumor-cell islands and free tumor cells. On the above basis, the view of selection may be inadequate for explanation of the development of the free-cell subline.

Next, the view of mutational changes of certain tumor cells should be taken into consideration. The results of observations indicated that a remarkable shift has occurred in the tumor cells of the AHF line not only in the chromosome number but also in the ideogram. There are present particular marker chromosomes, such as one metacentric chromosome of outstandingly large size and four large acrocentric chromosomes characteristic to the stem cell of the AHF line. The stem line ideogram of the AHF tumor has persisted unchanged since its development through many transfer generations: the AHF line was transferred serially in various strains of rats, such as WKA×AL, Buffalo, Wistar, Albany and Fischer, but the stem cell of this tumor has remained without a slight shift in every strain of rats.

From the above findings, it seems highly probable that mutational changes involving numerical and structural alterations of chromosomes had occurred in certain tumor cells forming a tumor-cell island (or islands), and that the mutated cells with genotypic changes might be superior in their competitive ability to others and give rise to the formation of the new subline. It is noticeable that the mutational changes of tumor cells had occurred during the transfer generations through WKA×AL rats, probably around the 67th generation, since the tumor cells showed to have the particular chromosome pattern after this transfer generation, together with the loss of cellular adhesiveness. It is then most probable that the concept of mutational changes of tumor stem-cells may be rather natural for the explanation of the rise of a new subline. Chromosomal changes have been found to occur in stemline cells during serial transfers resulting in the formation of a new tumor line in some other animal tumors (Makino and Sasaki 1958, Hauschka and Levan 1958, Hsu and Klatt 1958). Recently, Feldman and Sachs (1958), Ising (1955), Iversen (1958) and Koprowski, Theis and Love (1956) have reported that genotypic transitions have occurred in the stemline chromosomes during homologous or heterologous serial transfers of mouse and rat tumors. Quite recently, the present author has succeeded to establish another free cell hepatoma line which was produced after the methylcholanthrene treatment of AH-II tumor (Tanaka, unpublished). Levan and Biesele (1958) have stated that, through endoreduplication, translocation and so-called c-mitosis, the malignant cells may have a possiblity of altering chromosome morphology.

Summary

The present paper describes the cytological characteristics of a free-cell hepatoma

line which was produced from the chemically induced rat hepatoma of an island-type following transfers in hybrid rats.

The original ascites hepatoma II of rats had been maintained as island-type for 59 transfer generations in pure Buffalo rats from which the primary tumor was originated. After transplantation of this line into hybrid rats between Wistar-King A and Albany, tumor cells composing hepatoma-cell islands gradually lost their cellular adhesiveness and finally produced the free-cell population giving rise to a new subline, AHF.

Comparative studies have been made of the morphological and cytological characteristics of the original tumor, AH-II, and its subline, AHF. It has been shown that the lethal effect, transplantability and growth capacity of the AHF line are stronger than those observed in the AH-II line. Further, the tissue of the AHF line showed in part a sarcoma-like architecture that has not been seen in the tissue of the original AH-II.

The ideogram analysis indicates that the AHF clearly differs from the original AH-II both in chromosome number and other morphological aspects.

There is a possibility to show that the derivation of the AHF line seems to deal with mutational changes in tumor cell (or cells) of the original AH-II. Probably the mutated cells with genotypic changes might be superior in their competitive ability to others and give rise to the formation of the new subline.

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Explanation of Plate XXV

Fig. 1. General view of the AH-II tumor, showing small and compact island with smooth surface. ×100.

Fig. 2. Variability of succinic dehydrogenase activity in hepatoma-cell islands after several passages through WKA×AL rats. ×100.

Fig. 3. Tumor cells at the development of AHF, showing cells with elongated plasmatic protrusions. ×400.

Fig. 4. General view of AHF. ×400.

Fig. 5. Succinic dehydrogenease acitivity in tumor cells of AHF. ×200.

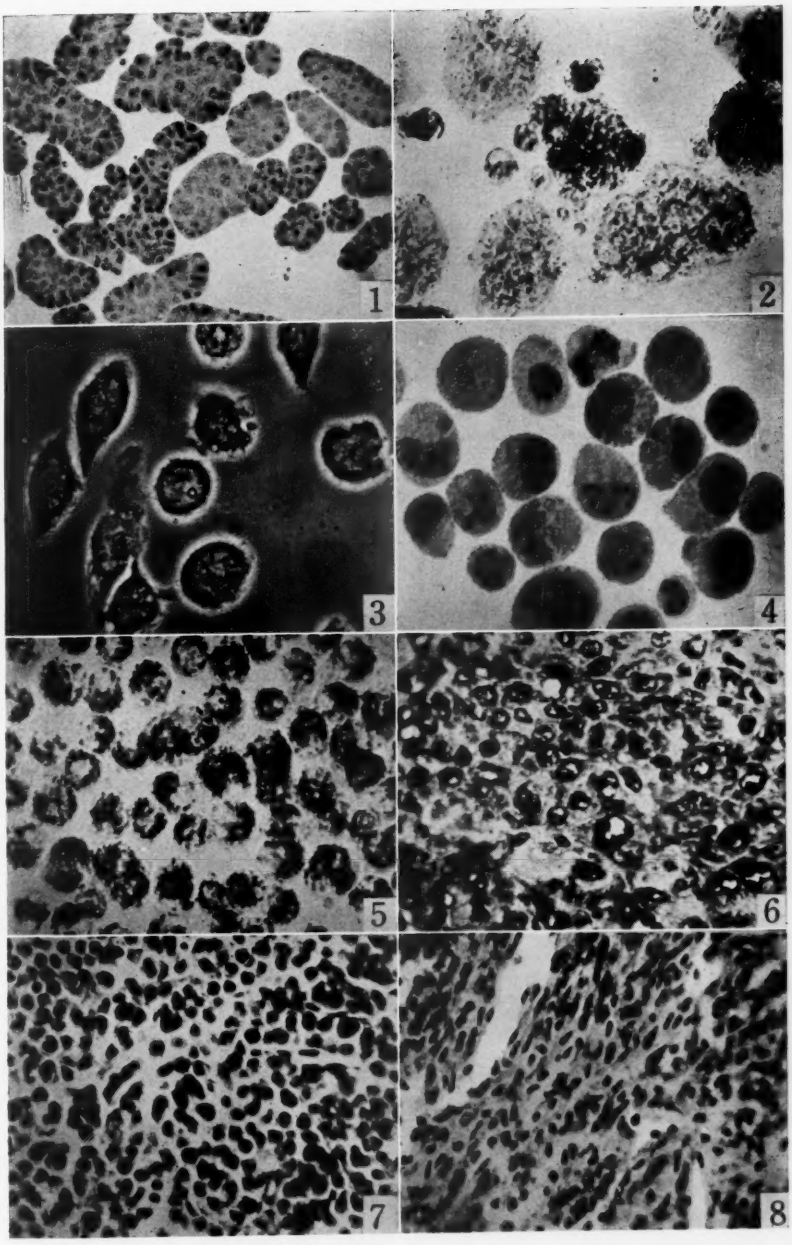
Fig. 6. A solid area of AH-II. Note variability in size of cells and neclei. ×400.

Fig. 7. A solid area of AHF, showing closely packed, unorganized round tumor cells in central zone of a tumor. ×300.

Fig. 8. A part of solid tumor of AHF. Note tumor cells showing a fusiform. ×300.

Explanation of Plate XXVI

Figs. 9-14. Photomicrographs of metaphase chromosomes. 9-11, original AH-II tumor showing 47 chromosomes. 12-14, subline AHF tumor, showing 38 chromosomes. ×1500.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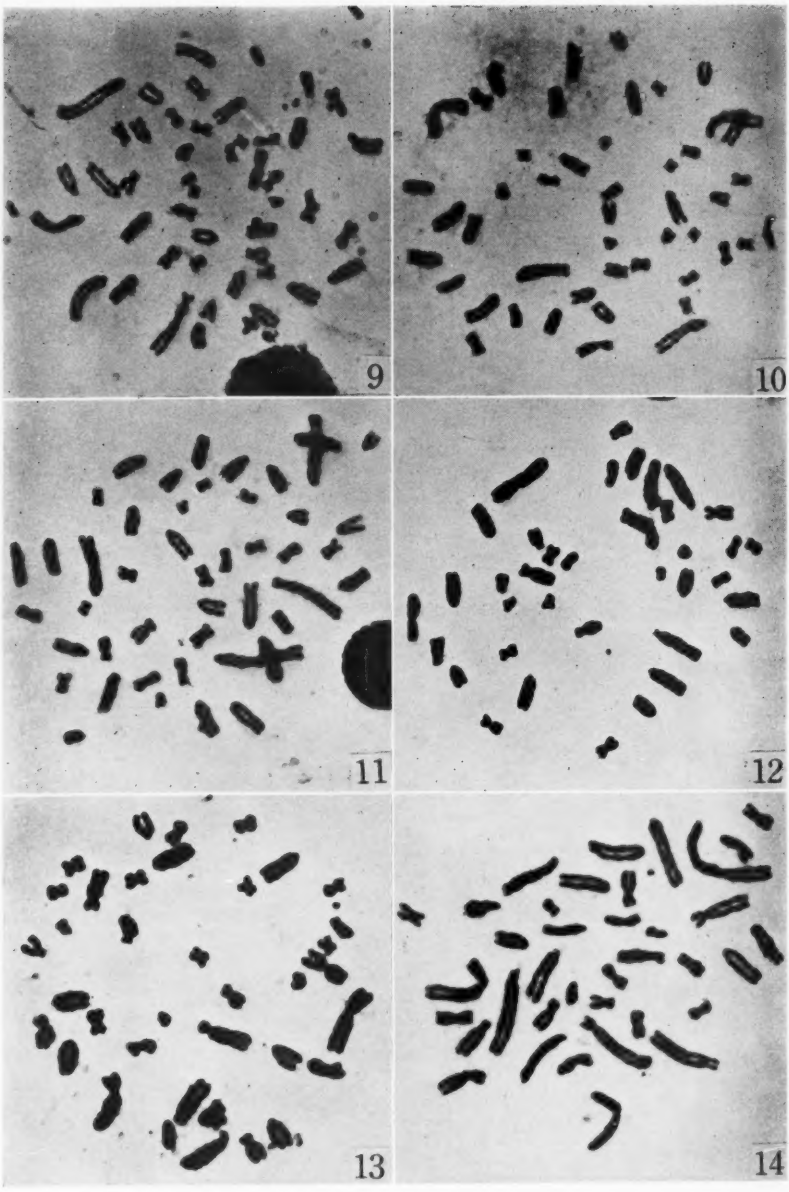
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[GANN, 50, 395~402; December, 1959]

CYTOLOGICAL STUDIES OF TUMORS. XXX. GENERAL CHARACTERISTICS AND CYTOLOGICAL FEATURES OF A TRANSPLANTABLE MOUSE TUMOR (EM TUMOR) OF SPONTANEOUS ORIGIN

(Plate XXVII)

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It has been shown that the transplantable tumors of rats and mice show a certain definite constancy by maintaining during serial transfer generations their cytological and neoplastic features that are especially characterized by a specific chromosome-number mode (or modes) together with a characteristic chromosome ideogram in tumor cells forming a stem-line (or -lines). However, the constancy of the stem-cell is not always stable, but under certain conditions, some alterations occur in the stem-line chromosomes of the tumor with its genotypic changes producing a shift in properties of the tumor (Makino 1957 a, b). It is therefore apparent that the genetic constitution of a tumor is closely correlated with the chromosome constitution of its stem cell.

The aim of the present study is to inquire into the change in chromosome condition of stem-line cells during successive transfers in the spontaneously developed, transplantable hypertriploid mouse tumor (EM tumor), and to transmit some information about the general histological features.

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Material and methods: The EM tumor here under study is one of the transplantable tumor lines in mice. This tumor was spontaneous in origin, and developed subcutaneously in the mammary area forming a nodular mass in a male mouse of the dilute cinnamon line belonging to the EM-mouse strain in the course of inbreeding. Pieces of the tumorous tissue from the primary tumor were crushed and injected in the subcutaneous tissue, or in the peritoneal cavity of new mice. Mice which had received

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the latter inoculation showed marked propagation of tumor cells in their peritoneal cavity as ascites tumors, while in the others the subcutaneous transplantations resulted in the formation of solid tumors. These tumor lines are referred to as 'EM tumor' in the following. Their serial transfers have been continued, since their origin in June 1957, for over 130 generations in the ascites line and for 85 generations in the solid one (January, 1959). For observations of the chromosomes in the ascites tumor, pretreatment with hypotonic sodium citrate solution (1.12% v/w) in combination with the squagh method with acetic orcein, according to Ford and Hamerton (1956), was adopted advantageously. For histological examinations, pieces of solid tumors fixed with Orth's solution were sectioned and subjected to the hematoxylin and eosin staining method.

General Characteristics of EM Tumor

(1) **Histological features of EM tumor:** Histo-pathological examinations indicated that the present tumor was primarily of mammary adenocarcinoma type. It showed metastasis into lymphatic nodes and mesentery in the peritoneal cavity. Preparations taken from the solid tumor after many transfers showed numerous acinar or glandular structures which consist of tumor cells dividing actively (Fig. 6) and forming parallel strata in the proximal region of the implant. The spaces in contact with or between the strata and muscular tissue were filled with stromatous cells. Tumor cells showing active mitosis infiltrated irregularly into the neighboring tissues. It is then evident that the solid EM tumor showed an undifferentiated and highly malignant growth at the site of transplantation.

(2) **Cytological features of EM tumor in ascites form:** As described above, the ascites form of the present tumor was established in new mice by the intraperitoneal injections of crushed tumorous masses after treatment with a 0.3% trypsin solution. The tumor cells showed active proliferation in the peritoneal cavity of the host. They were capable of transplantation without any special treatment. Temporary smear preparations showed that tumor cells were mostly round in outline having one, or rarely two, nuclei (Fig. 7). The nuclei were oval or kidney-like in shape. The nucleoli varied in number from one to three or more. Some cells possessed a cytoplasmic protrusion like a spine showing in extreme cases a dumb-bell-shape in one end of the cell membrane. The cells of this type were nearly constant in occurrence showing a range of 30 to 50 per cent in all samples from the same transplant generation. Though the role of this protrusion remains unknown, it may take part in the adhesiveness of the cell and/or in intercellular connection important for invasion. Later in the period after transplantation a large amount of small vacuoles and particles like oil-droplets were visible under the cell surface when observed in living state. Mitotic behavior was regular in general, though the chromosomes were very

sticky in early transfer generations.

(3) **Transplantability and the survival of tumor-bearing animals:** Since its origin, transmission of the present tumor has been carried out in mice of EM strain. No mouse from the substrains such as EM-black, -cinnamon, -dilute cinnamon, -agouti, -dilute agouti and -albino (Sato 1957) showed any remarkable difference in lethal transplantability for this tumor. Lethal transplantability was generally more than 98 per cent in the ascites line and 95 per cent in the solid one.

The life span of the animals bearing the present ascites tumor was relatively constant, being 10.5 days in an average, with a fluctuation ranging from 6 to 15 days. The life days of the animals bearing the solid tumor varied rather widely from 6 days to more than 7 weeks with a mean life span of 14.2 days.

Positive results of transplantability for mice of foreign strains were also obtained by the use of SM, C 57 BL and D-240 mice. No significant difference could be detected between these mice and EM mice with respect to transplantability of the tumor and life span of the animals.

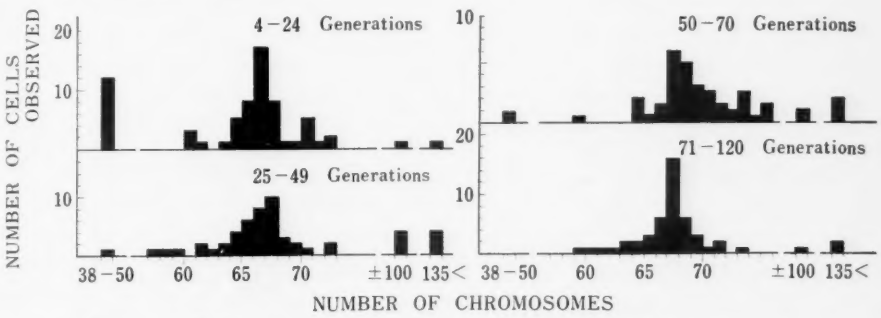


Figure 1. Changes in chromosome number distribution in EM ascites tumor during serial transfer generations.

The stemline Chromosome Pattern

(1) **Chromosome number:** Since the chromosomes showed at metaphase strong stickness during a period from the first to third generation of successive transfers, no sufficient analysis of chromosomes was made in those generations. The chromosomes were studied in the samples from the ascites tumor. Pretreatment with a hypotonic sodium citrate solution was advantageous to obtain preparations showing well-spread metaphase chromosomes available for observation. Chromosome counting was carried out in 50 or more reliable metaphase plates in the samples taken from the 4th to 24th, the 25th to 49th, the 50th to 70th and the 71st to 120th generations, respectively.

In earlier samples derived from the 4th to 24th transfer generations (July to Septem-

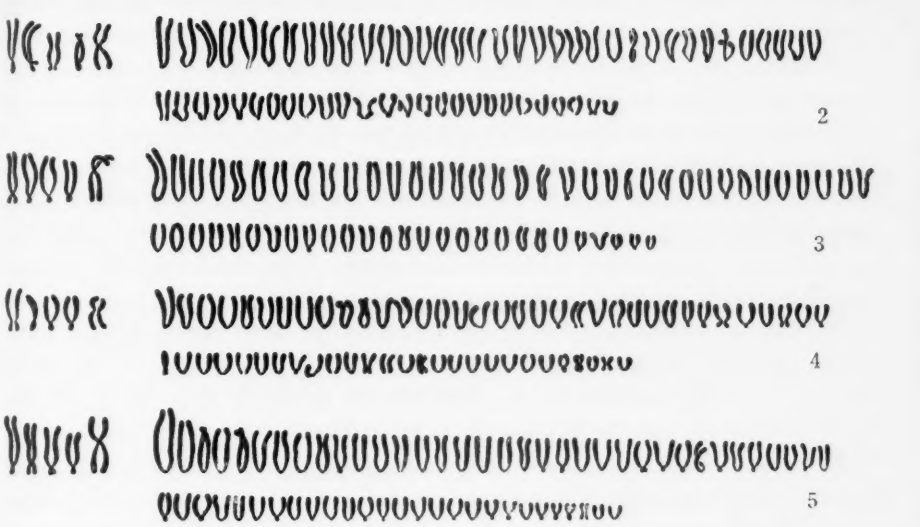
ber 1957), the variation of the chromosome number ranged from 60 to 72 with the modal number at 66; 27.4 per cent of cells observed fell within the near-diploid range, varying from 38 to 50 in number with a mode at 41. The cells having 41 chromosomes showed a pronounced decrease in the later transfer generations (Fig. 1).

Gradual shift of the modal chromosome-number occurred with the increase of transfer generations. The modal number was found to be 66 in the period from the 4th to 24th generations (July to September, 1957), while it showed a shift to 67 in the period from the 25th to the 70th transfer generations (October 1957 to April 1958). For details one may refer to the data given in Figure 1. Along with the above change, the decrease in number of cells lying in the near-diploid range was striking. It is interesting to note the occurrence of minute chromosomes varying in number from one to three in metaphasic cells (Fig. 10). They are dot-like in outline, and in length about two-thirds that of the smallest chromosome. These minute elements take part in the fluctuation of the chromosome number around the modal number, 67.

In recent samples taken from the 71st to 120th transfer generations (April to December 1958), the frequency variation of the chromosome number in tumor cells came to fall within a rather narrow range with a unimodal value at 67 in a frequency of 32.7%, as shown in Figure 1. It is remarkable that the disappearance of minute chromosomes had occurred in tumor cells from these samples, with a striking decrease in number of cells of the near-diploid range.

From the results of the above observations, it is evident that a numerical shift of the chromosomes from 66 to 67 occurred in the stem cells, and further that the numerical variations observed in the 25th to 49th transfer generations and in the 50th to 70th transfer generations seem to represent a transitional process. In the recent transfer generations, the chromosome numer, 67, has persisted in a stable condition as the stemline number (January 1959). It is therefore apparent that the present tumor is of hypertriploid type.

(2) Morphology of the chromosomes: The chromosomes of the stem-cells of the present EM tumor in ascites form were analysed on the basis of five metaphasic cells in each sample from the 8th, 25th, 50th and 74th transfer generations. Four representative ideograms derived from the above four transfer generations are shown in Figures 2 to 5. The cells from the 8th generation contain 66 chromosomes (Fig. 2), while those from the other generations show uniformly 67 chromosomes (Figs. 3-5). The stem cells of EM tumor are clearly distinguishable from the somatic cells by their content of a certain number of rod-like, V- and J-shaped chromosomes. Detailed analysis revealed that in every cell observed a constant occurrence of four large J-shaped chromosomes of subterminal structure and a V-shaped element of remarkable size are striking in the set because of their configuration clearly distinguishable from the others. The remaining 62 elements are of a rod-type in general appearance,



Figures 2–5. Ideogram analyses of EM ascites tumor. Fig. 2, 66 chromosomes, from the 8th generation. Fig. 3, 67 chromosomes, from the 25th generation. Fig. 4, 67 chromosomes from the 50th generation. Fig. 5, 67 chromosomes, from the 74th generation.

though there are certain elements suggesting a subterminal nature; they show a gradual order in diminution of size. Comparison of the stemline ideograms revealed that there is no visible morphological difference in the chromosomes shown in Figures 2 to 5, though Figure 2 contains one rod-type chromosome less than there are in Figures 3 to 5.

Based on the above findings, it can be concluded that the stemline chromosome pattern of the present EM tumor clearly differs from that of the normal tissue both in numerical and morphological aspects. It is very probable that the chromosomes of the tumor show inner changes involving structural rearrangements and small adjustments that may correlate with the rise of the neoplastic condition (Levan 1956a, Makino 1957a, b, Makino and Sasaki 1958). Attention should be called to the occurrence of minute chromosomes, one to three in number; they were found commonly in the samples from earlier generations, but disappeared in those from the later generations.

Discussion

Recent extensive studies on the chromosomes of rat and mouse ascites tumor have revealed a very pronounced constancy of the stemline chromosomes (Levan 1956 a,

Makino 1957 b). Considerable evidence derived from experiments making use of drastic applications, cold storage and some procedures strongly supports the above view (Makino 1957a, b). The stability of the stemline chromosomes, however, is not always permanent. In the Yoshida sarcoma, Makino and Sasaki (1958) observed numerical and structural changes of chromosomes in the stem cells during the course of successive transfers, reporting that such changes produced shifts in property of the tumor resulting in the rise of new sublines. Makino (1957c) also reported that a hypotriploid stemline of the MTK-IV tumor started from the hypotetraploid line through gradual elimination of certain chromosomes during serial transfers. According to Tonomura and Sasaki (1957), the stemline chromosomes of the MTK-sarcomas II and III had undergone morphological and numerical shifts during several years' serial transfers. Further, Sasaki (1958) studied the rise and further development of the rat ascites hepatoma (H-17), reporting that the numerical shift of the stemline chromosomes occurred in close association with changes in the neoplastic properties.

The results of the present investigation have shown that in the EM ascites tumor considered here a transition of the stemline chromosome number had occurred during successive transfers. In earlier transfer generations the stem cells were characterized by 66 chromosomes, while in later generations the cells having 67 chromosomes formed a stemline with a predominat occurring mode.

Morphological analysis of the stemline ideogram of this tumor has shown that there is no remarkable morphological difference between the stem cells having 66 chromosomes and those with 67 chromosomes (Figs. 2-5). It is interesting to see that no appreciable difference has occurred in transplantability of the tumor nor in the life span of the tumor-bearing animals during the course of transitional changes of the chromosomes.

Makino (1957a) has argued the tendency of neoplastic cells to undergo a transition after their origin, stating that some adjustments or mutational changes of certain chromosomes might lead to genotypic changes of the neoplastic population, and that such shifts cause the changes in properties of the tumor which result in the rise of a new tumor line. In the EM tumor, the shift of the chromosome number from 66 to 67 occurred in the hypertriploid modal cells during successive transfers. At present, it is impossible to state with certainty that this alteration was caused by the addition of a minute chromosome, or induced from the selection of the cells possessing 67 chromosomes and their predominant propagation.

Summary

The present article deals with histological and cytological studies of the EM tumor of mice, spontaneous in origin and transplantable in both ascites and solid forms. Originally the tumor developed subcutaneously in the mammary area of a male mouse

of EM strain. Through subcutaneous and intraperitoneal inoculations of cell suspension obtained from the primary tumor into new mice, both the solid and the ascites tumor lines were established which have been maintained for 85 transfer generations in the solid line and 130 transfer generations in the ascites line.

Histo-pathological examinations revealed that EM tumor of the solid line was a type of mammary adenocarcinoma, showing malignant growth at the site of transplantation. Temporary smear preparations showed that the tumor cells in the ascites line were generally similar than those observed in most of the other ascites tumors of rats and mice.

The results of the chromosome study of the present tumor in ascites form showed that a numerical shift of the chromosomes from 66 to 67 occurred in the stem cells during the course of serial transfer generations, while the number of 67 was found as a stable stemline number in the later generations. The ideogram of this tumor is clearly distinguishable from the normal ideogram in that it shows a constant occurrence of four large J-shaped chromosomes, a V-shaped element of remarkable size and the remaining 62 rod-type elements. Minute chromosomes, one to three in number, occurred in samples from earlier transfer generations, but disappeared in those from later generations. Generally they take part in the variation of the chromosome number around the modal number.

Lethal transplantability of the present tumor was of more than 98 per cent occurrence in the ascites line and 95 per cent in the solid one. The life span of the animals bearing EM tumor in the ascites line was 10.5 days on the average, while that of the animals bearing the solid one varied rather widely from 6 days to more than 7 weeks with a mean life span of 14.2 days. No significant difference could be detected between EM mice and foreign strain mice such as SM, C 57 BL and D-240 mice with respect to transplantability and life span. Neither was appreciable difference found in transplantability of the tumor during the course of transitional changes of chromosomes.

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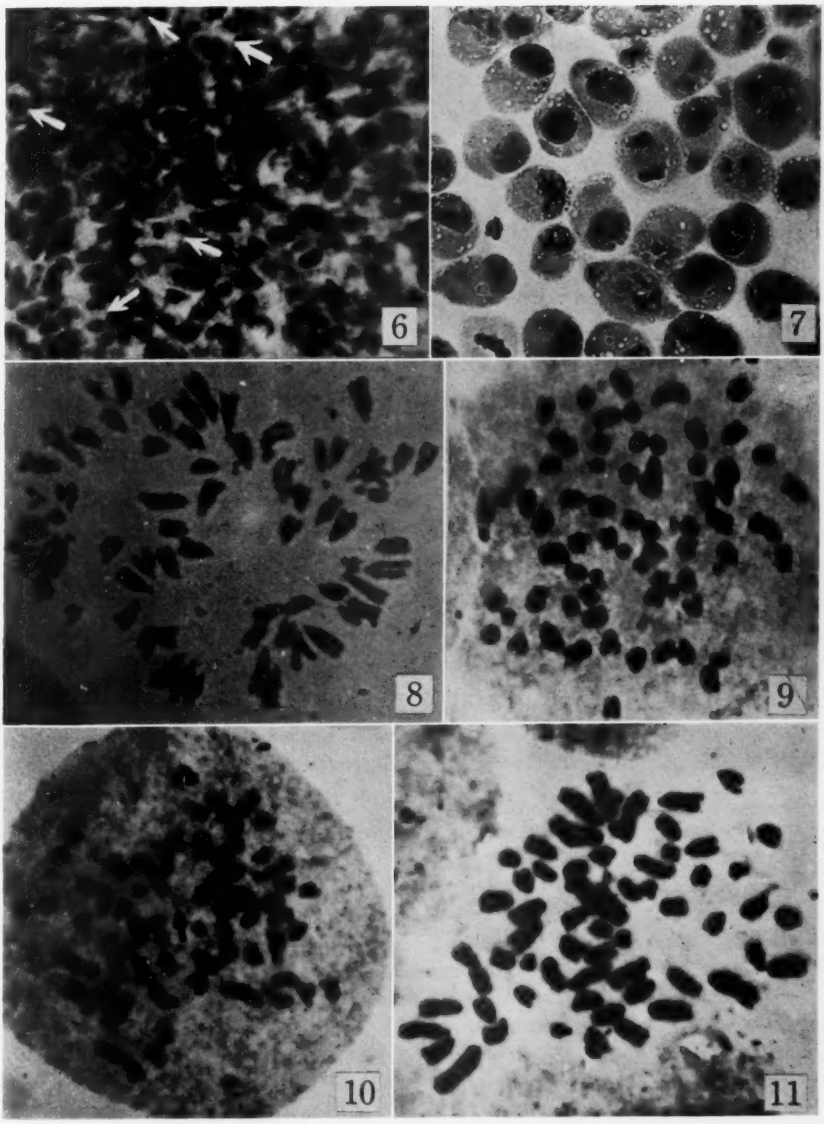
Explanation of Plate XXVII

Fig. 6. Histological structure of EM solid tumor. Arrows indicate tumor cells in mitosis: hematoxylin and eosin preparation, from the 30th transfer generation, ×300.

Fig. 7. Photomicrograph showing general appearance of EM ascites tumor, from the 70th generation, 5 days after inoculation. Smear preparation with acetic dahlia, ×500.

Figs. 8–11. Photomicrographs of metaphase chromosomes of EM ascites tumor. Fig. 8, 66 chromosomes, from the 8th generation, ×1800. Fig. 9, 67 chromosomes, from the 25th generation, ×1500. Fig. 10, 67 chromosomes, from the 50th generation. Arrow indicates a minute chromosome, ×1500. Fig. 11, 67 chromosomes, from the 74th generation, ×1800. Fig. 11 corresponds to Fig. 5.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[GANN, **50**, 403～408; December, 1959]

CYTOLOGICAL STUDIES OF TUMORS. XXXI. A CHROMOSOME STUDY IN A HUMAN GASTRIC CARCINOMA

(Plate XXVIII)

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The data at hand derived from some recent investigations of the chromosomes of human tumors have shown that the most frequently occurring tumor cells which have a characteristic number mode (or modes) along with a particular chromosome ideogram (or ideograms) form a stem-line (or -lines) of tumor cells which are the primary progenitors of growing neoplasms, in parallel with the evidence presented by the chromosome analysis of rat and mouse ascites tumors (Hasen-Melander, Kullander & Melander 1956, Levan 1956, Ising & Levan 1957, Manna 1957, Wakabayashi & Ishihara 1958, Makino, Ishihara & Tonomura, 1959). Since the chromosome conditions of human tumors are important in the consideration of the general clinical and pathological properties of neoplasms, the karyological data of human tumors have strikingly aroused a great deal of interest in the medical field.

The present paper reports the results in some detail of a chromosome analysis in tumor cells of a human gastric carcinoma in ascites form, with special regard to certain chromosomal features in relation to the therapeutic data.

The author wishes to tender his expression of sincere gratitude to Professor Masaru Wakabayashi and Professor Sajiro Makino for their direction and valuable advices. Further cordial thanks are offered to Dr. Kouichi Kaneta, Department of Radiology, Sapporo Medical College, for the material with which the present study was carried out.

Method: The ascites material for study was obtained from a patient with a gastric carcinoma in the Hospital of Sapporo Medical College. The tumor cells were obtained by centrifuging the ascites for ten minutes at 700 r.p.m. They were smeared on slides and stained with acetic dahlia according to the water pretreatment method (Makino 1957).

Records of the tumor patient: The patient who provided the present material was a man, 62 years old. On the 20th of July, 1957, he was diagnosed to have a gastric carcinoma, and laparatomy was performed on the 31st. On the 10th of December 1957, metastasis of the tumor was observed on the operated portion. Radiation therapy was carried out with 5400r irradiation, from February 7 to March 9, 1958. In the middle of February the patient showed a considerable amount of ascites. The ascites was taken out by paracentesis on the 8th of March. Microscopical examinations of the ascites showed a large number of dividing tumor cells. On the 17th of March, another metastatic tumor was found in the lower abdomen. The X-ray therapy was done toward the metastatic tumor by spot irradiation with 3400r during a period from

March 24 to May 7. The patient died on the 25th of May. No histological diagnosis of this tumor was made since no autopsy was carried out.

OBSERVATIONS

1) **Tumor cells:** The peritoneal fluid contained a large number of tumor cells of various sizes, their shape being round or oval. The mitotic rate of tumor cells was about 2.0 percent. Most of the mitotic cells were characterized by normal mitotic behavior, though a few showed mitotic abnormalities such as stickiness and coalescence or condensation of chromosomes.

2) **Chromosome number:** For the chromosome counting, 201 good metaphase plates which showed clear chromosomes available for counting were selected from three different samplings. The results are summarized in Table 1.

Table 1. Chromosome number distributions in three different samplings in a patient with a gastric carcinoma.

Sampling	Chromosome number																						
	26	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
(I) March 7		1	1					4	8	7	11	28	5	1	3	2				1	2	2	
(II) March 24	1	1					1	6	28	3	5	9	4		1		1		1				
(III) May 10							2	2	7	2		2	1										

Sampling	Chromosome number																									Total
	57	58	64	65	68	70	74	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	93	94	95	
(I) March 7		1		1		1	1				1	1	2	6	3	4						1	1	1		100
(II) March 24					1			2	1	1	1	3	1	2	2	3	1		2	1	2				1	84
(III) May 10			1																							17

In the first sampling made on March 7 (1958), the chromosome number exhibited a wide range of variation from 35 to 94, showing two definite modes: one modal range fell between 41 and 48, the modal number being 45 (28 percent), while the other between 80 and 84 (16.0 percent).

In the second sampling on March 24, the chromosome number varied from 26 to 95, showing two modes as in the first sample. In this sampling, however, the tumor cells with 45 chromosomes (45-cells) which ranked first in the first sample, showed remarkable decrease (10.8 percent), whereas the tumor cells with 42 chromosomes (42-cells) were observed most frequently (34.1 percent). The tumor cells with 80-84 chromosomes (80-84 cells) were observed to number about the same as in the first sampling (13.09 percent).

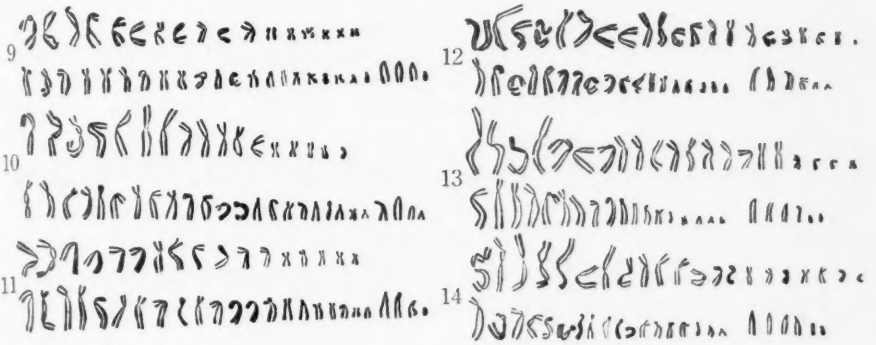
In the third sampling on May 10, the chromosome number varied from 40 to 60. As in the second sample, 42-cells occurred with the highest frequency (31.2 percent), while the 45-cells appeared with such a low frequency as 10.7 percent.

On the basis of the above results, it is possible to state that there are in this tumor three cell-populations which form three cell-lines, two at near-diploid and one in the range from hypertriploid to hypotetraploid.

3) **Chromosome ideogram:** The chromosomes were morphologically classified into three groups according to Tjio and Levan (1956) as follows: M (the chromosome with a median-submedian centromere), S (the one with a subterminal centromere) and T (the one with a terminal centromere). The individual chromosomes in each group were arranged in oder of size.

Ideogram analyses were made in eight ideal metaphase plates of 42 and of 45 cells, and in five of 80–84 cells.

42-cell: The length of chromosomes of 42-cells fell mostly between 2 and 11μ, the largest being 15μ. The 42-cell was composed of approximately 17 M, 21 S and 4 T chromosomes in a formula, 17M+21S+4T (Figs. 1, 2, 9, 10, 11). Every 42-cell in three different samplings showed quite identical chromosome patterns.



Figs. 9–14. Serial alignments of chromosomes of a human gastric carcinoma. Figs. 9–11; 42-cell line. Figs. 12–14; 45-cell line.

45-cell: In the 45-cell plates chromosomes varied from 1.9μ to 13.5μ in length. Chromosomes from No. 1 to No. 4 were rather large over 10μ long. The ideogram was made up of approximately 21 M, 18 S and 6 T chromosomes showing a formula, 21M+18S+6T (Figs. 3, 4, 12, 13, 14).

80–84-cell: The 80–84-cell was constituted by about 34 M-chromosomes, 40–42 S-chromosomes and 8 T-chromosomes having a formula of 34M+40-42S+8T. It is evident that the 80–84-cell has almost twice as many chromosomes as those of the 42-cell (Figs. 5, 6, 15, 16).

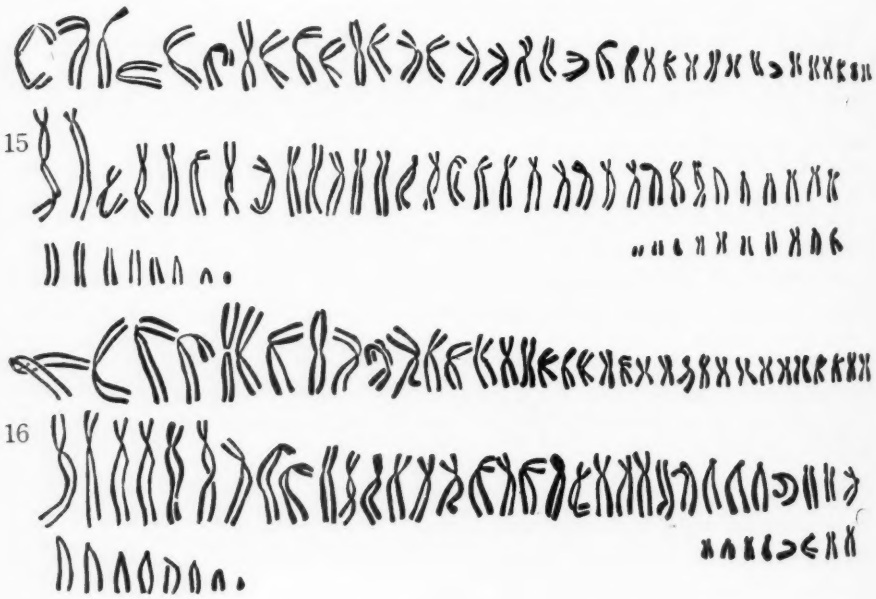
Thus it was found that each stem-line had its own characteristic chromosomal complex clearly different from each of the others. None, of course, had any similarity

to human normal chromosomes in number and morphology, which, according to Tjio and Levan (1956) and Makino and Sasaki (1959), are 46 in number and consist of 20 M, 20 S and 6 T chromosomes.

An interesting feature was presented that in some cells the chromosomes showed a sign of endoreduplication (Figs. 7, 8). Such cells were observed in about 2 to 3 percent of mitotic cells under study. In reference to the fact that the 80–84–cell has a constitution which is approximately double that of the 42–cell, it seems very probable that the former might have been produced by means of endoreduplication from the 42–cell. Levan and Hauschka (1953) reported in several mouse ascites tumors that some hyperploid tumor cells were formed by endoreduplication of chromosomes.

Discussion

The results of the present observations have shown clearly that the gastric carcinoma here considered is characteristic in having three populations of tumor cells, two at near-diploid and one in the range from hypertriploid to hypotetraploid; they show a high frequency in occurrence and possess a particular chromosome pattern in each. In parallel with the conclusions derived from rat and mouse tumors, it seems likely that the three populations of tumor cells represent three stem cell lines



Figs. 15–16. Serial alignment of chromosomes in 80–84 cell line of a human gastric carcinoma. Fig. 15; 84–cell. Fig. 16; 82–cell.

in separate existence which serve as primary progenitors in growth of this tumor. It seems probable that two cell-lines consisting of 42-cells and 45-cells are the principal lines. The line of 80-84-cells which is less remarkable than the above two is regarded as a supplementary stem-line, since the cells with 80-84 chromosomes seem to be produced through endoreduplication of chromosomes in the 42-cell.

It is of great interest to find that the two cell-lines, 42-cells and 45-cells, showed different frequencies in three samplings: 45-cells occurred with the highest frequency in the first sampling, whereas in the second and third samplings, 42-cells appeared most frequently. The 42-cells which appeared in the second and third samples seem not to be new products, since they were quite the same in chromosome constitution in all three samplings. The evidence presented may be reasonably explained as due to adaptability of the two cell-types to the physiological conditions of the patient: the 42-cells are probably more adaptable than the 45-cells to a new condition of the patient caused by the X-ray therapy.

In the Hirosaki sarcoma Makino and Kanò (1953) have reported the occurrence of five types of tumor cells which vary in frequency with the increase of transfer generations. Watanabe and Tonomura (1955) have found in the Watanabe ascites hepatoma that there are three stem-lines which show a change of frequency during transfer generations. Further, Hasen-Melander, Kullander and Melander (1956) working on a human ovarian cytocarcinoma have reported a change of frequency in two types of srem-line cells in different samplings.

It is interesting that both the 42-cells and 45-cells are characterized by some large chromosomes, 10 to 15μ in length: especially, No. 1 to No. 4 chromosomes of 45-cells measure over 10μ in length. The largest chromosomes of the human normal cell was reported to be 8 to 10μ by both Tjio and Levan (1956) and by Chu and Giles (1958).

In addition to the difference in length of the chromosomes, three types of stem-cells greatly differ in both chromosome number and constitution from normal somatic cells. Each type possesses its own characteristic constitution of M-, S- and T-chromosomes completely different from each other. Thus it appears that, together with the numerical changes, considerable structural and mutational changes might have taken place in the tumor chromosomes in relation to the development of the tumor. Such a cytological situation as observed in this gastric carcinoma is comparable to that found in certain transplantable animal tumors (Makino 1957).

Summary

The present paper describes the results of a chromosome analysis in tumor cells of a human gastric carcinoma. The samples for study were obtained from the peritoneal fluid of a patient.

It was found that there were present in this tumor three populations of tumor cells which were characterized by the chromosome numbers of 42, 45 and 80–84, each showing a particular chromosome pattern and a high frequency in occurrence. They are regarded as stem-lines of tumor cells which contribute principally to the growth of this tumor: the lines formed by 42- and 45-cells are considered to be principal lines and the line of 80–84 cells is supplementary.

Evidence was obtained that the frequency in occurrence of the two principal stem-lines varied in three samplings made on different dates. The variation seems explicable as due to the adaptability of the cell-types to certain physiological conditions of the patient.

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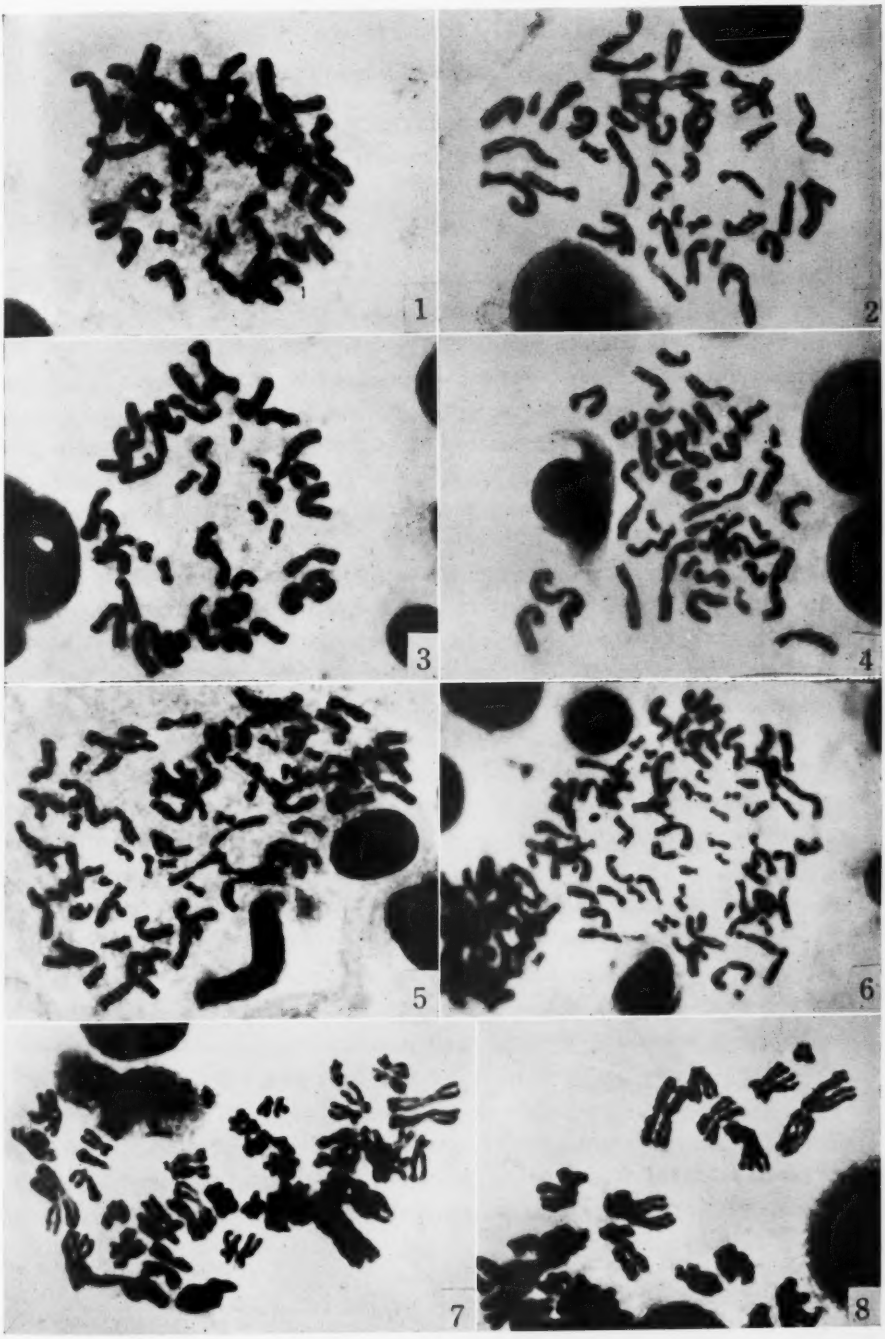
Explanation of Plate XXVIII

Figs. 1–6. Microphotographs of chromosomes of tumor cells in a human gastric carcinoma. Figs. 1–2; 42-cell line. Figs. 3–4; 45-cell line. ×1600. Figs. 5–6; 84-cell line. ×1150.

Figs. 7–8. Microphotographs indicating endoreduplication of chromosomes. ×1600.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[GANN, 50, 409~415; December, 1959]

ON THE DUODENAL SPREAD OF GASTRIC CANCER

(Plates XXIX—XXXII)

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INTRODUCTION

The adequate removal of the cancer of the stomach is difficult because of deficient knowledge as to the nature of its spread. Studies on metastasis of gastric cancer have been reported by various authors (1-4). Jinnai has indicated that the cancer consisting of smaller cancer cells is metastasized more frequently and reaches more distant lymph nodes than in the case of the metastasis of the cancer consisting of larger cancer cells (5). On the histological observations of cancer tissues, Imai has established the CPL classification on the mode of cancer growth (6-8), and the classification is said to be available in the determination of the prognosis of a cancer patient (9).

In 1861 Rokitansky laid down the law that the pyloric cancer was exactly bound by the pyloric ring, and never reached beyond that to the duodenum (10). It is described in some text-books of surgery and pathology that the growth of stomach cancer has no tendency to invade the duodenum (11). However, after performing gastrectomy for stomach cancer, various authors have indicated the recurrence at duodenal stump (12-15). It is noticed that the duodenal extensions of gastric cancer occured in various degrees and the extension reached several centimeters according to the observations from either surgical or autopsy materials (16-18). Reviews of the literatures including the study on the duodenal spread of gastric cancer were given by Castleman, Fodden and Zinninger (16-18).

In the present paper, the duodenal spread of gastric cancer is discussed on the observations from autopsy materials and on the experiments on rabbits.

I. Observations on autopsy materials

The materials in this study included 27 cases of gastric cancer autopsied at the Department of Pathology, Nara Medical College, of which 22 were of gastric cancer without gastrectomy and 5 with gastrectomy.

A) Observations on the cases of gastric cancer without gastrectomy

Materials and Methods: Twenty two cases of gastric cancer were studied, of which 12 were of the cancer of the pylorus, 5 were that of the body, 3 that of the cardia and 2 that of the diffuse type. In these cases, the site, form, metastasis and the extension

of the cancer were examined macroscopically. The duodenal spread of gastric cancer were studied on the specimens which were taken crosswise to the long axis of the duodenum from 1 cm to 3 cm distal from the pyloric ring.

Results:

1) On the pyloric cancer. The cancerous invasion into the duodenum were found in 5 cases out of 12 pyloric cancers. In 4 cases of them the invasion was macroscopically visible and 1 case showed only microscopic invasion. Macroscopically, the involved duodenal mucosa showed plauqe-like thickning or ulceration, and no sharp margin could be seen between the mucous membrane of the stomach and of the duodenum. Microscopically, the invasions of cancer were found within all layers of the duodenum, especially remarkable within the submucosa and subserosa. The cancer cells were chiefly noticed in the lymphatics (Fig. 1, 3) and occasionally the extension was revealed by continuous infiltration (Fig. 2). Case No. 138 showed the clumps of cancer cells in the lymph vessels of the mucosal villi. The invaded cancer tissues showed solid or adenocarcinomatous or scirrhous pattern.

The gastric cancers with duodenal spread were macroscopically ulcerative or diffuse infiltrative and not polypoid in type. In these cases, the metastatic involvement of perigastric lymph nodes was remarkable, and the extensions of cancer along the both curvatures were prominent. Microscopically, these cancers showed the P or L Form by Imai's Classification.

2) On the cancer of the body of the stomach. Two cases out of 5 cancers of the body showed microscopic invasion into the duodenum. In case No. 180, the invasion was found within all layers of the duodenal wall, especially in submucosal (Fig. 4) and subserosal lymphatics. In case No. 253, the invasion revealed continuous growth of cancer in subserosal layer (Fig. 5). These gastric cancers which had the duodenal spread, macroscopically showed large ulcerative or diffuse pattern, however, mucosa of the antrum of the stomach was not involved. The metastasis of cancer in the perigastric and hepatic lymph glands was significant, and large congromerated lymph nodes were obtained. In case No. 180, the gastric cancer showed remarkable extension along the lesser curvature, and in case No. 253, the extension along the greater curvature was prominent.

3) On the cancer of the cardia of the stomach. Microscopic invasion into the duodenum was noticed in 2 cases out of 3 of the cancer of the cardia. These invasions were revealed by the presence of cancer cells in the lymphatics within the duodenal subserosa (Fig. 6).

Macroscopically, these gastric cancers which had duodenal spread, showed a large ulcerative pattern, and the cancerous involvements along the lesser or greater curvature were manifest. In the antrum of the stomach, the mucosa was not involved macroscopically, but the extensive lymphatic spread of cancer cells was microscopi-

cally noticed within the subserosal and the submucosal network of the lymphatics. The peripyloric glands were involved with cancer and showed large congromerates.

4) On the diffuse cancer of the stomach. In 1 case out of 2 of diffuse cancer, the duodenal spread was microscopically found in the muscle and subserosal layer, although macroscopically the duodenal wall was not involved. The extension was revealed by direct continuous growth of cancer cells in the tissue cleavage (Fig. 7). This stomach cancer was grossly scirrhous, and showed extension along the lesser curvature and metastasized in the periplyloric glands. Microscopically, the cancer tissue showed adenocarcinomatous pattern.

B) Observations on gastric cancer with gastrectomy

Materials and Methods: Five cases of gastric cancer with gastrectomy were examined macroscopically, andthe tissues were taken from the remaining stomach, oral stump of the duodenum, and the jejunum near the operative junction of the stomach and the jejunum, and these tissues were microscopically studied by hematoxylin and eosin staining.

Results:

Cancerous involvements in remaining stomach wall were noticed in 3 cases out of 5 of this series. In these 3 cases, the cancer cells invaded the wall of the jejunum for several centimeters beyond the junction of the stomach and jejunum (Fig. 8).

In 2 ceses out of the above 3, the microscopic invasions of cancer cells were noticed in muscular and subserosal layer of the duodenal stump (Fig. 9), although no cancerous masses were noticed macroscopically in the stump.

II. Experiments concerning the lymph flow in the pyloric region of the rabbits

Materials and Methods: Male and female rabbits, weighing about 1 kg, were used. Case No. 1 had 0.1cc of 3 per cent of Indian Ink injection into the gastric submucosa, about 1 cm proximal from the pyloric ring along its lesser curvature. Case No. 2 had a similar dose of Indian ink injection into the subserosa of the stomach wall at the same portion as the above. In case No. 3, 0.3 cc of the ink was injected in all layers of the stomach at the same portion as the above. In case No. 4, the same dose of the Ink was injected in all layers of corpus of the stomach.

These rabbits were sacrificed 5 days after injection. The stomach and the duodenum were examined were macroscopically, and were fixed in formalin. After fixation, the tissues were taken longitudinally from the portion, including the injected gastric wall with duodenal wall 3 cm distal from pylopic ring. These tissues were enbedded in paraffin and stained with hematoxylin and eosin for microscopic examination.

Results: In these experiments, it is noticed that the Indian Ink injected into the gastric wall was discharged through the lymph shannels in the subserosa toward the regional lymph nodes. But the flows of the Ink through the lymph channels never

reached the duodenal subserosa.

Within 5 days after injection, Indian Ink were recognized at the injected portion in gastric wall, and in enlarged lymph nodes in all cases. In case No. 1 and 2 in which small amount of Indian Ink had been injected into the gastric wall near the pyloric ring, it was observed that the injected Indian Ink had reached the pyloric ring, but not extended into the duodenal wall beyond the pyloric ring. In case No. 3 which had been injected large amount of Indian Ink in the gastric wall near the pyloric ring, the injected Ink extended grossly into the subserosa and submucosa of the duodenum about 1 cm distal beyond the pyloric ring (Fig. 10, 11). Microscopically, many macrophages with carbon particles were noticed in subserosa and in submucosa of the duodenum, occasionally in the lymphatics within such layers (Fig. 12). In case No. 4 which had Indian Ink injection in the corpus of the stomach, Ink was not extended into the duodenum.

Discussion

From the observations on autopsy materials, it was demonstrated that about one-half of the cases of gastric cancer showed the duodenal invasion microscopically, and main lesion of those cancers existed in nearly every part of the stomach. Macroscopically those gastric cancers showed ulcerative or diffuse infiltrative form. Histological types of the gastric cancer had no relation to the frequency of the duodenal spread.

The involved cancer cells were chiefly found in the lymphatic plexuses or spaces within the subserosa or submucosa of the duodenum. These findings revealed that the duodenal spread of gastric cancer has not been caused by dissemination of cancer cells on serosa of the duodenum, but may have been caused by lymphatic permiation through the lymphatic plexuses or continuous extension through the lymphatic spaces. The mode of duodenal invasion of pyloric cancer has been well described by Fodden (17). However, it is not established whether there may exist free communication between the lymphatic plexuses of gastric wall and that of the duodenal wall. Fodden had indicated that anatomical pathways existed in plenty between the two viscera, proved by his experiment and the natural migration of cancer cells. Kihara had point out the presence of "Extravasculäres Saftbahnsystem (Kihara)" within the loose connective tissue everywhere (19). From these opinions, it is supposed that there may exist lymphatic communication between the two viscera, as reported by some authors (20). Our supplementary experiments revealed that when small amount of Indian ink was injected into the gastric wall near the pyloric ring, the ink was not extended into the duodenal wall beyond the pyloric ring. However, when fairly large amount of Indian ink was injected at the same portion as the above, it was noticed the ink extended into both submucosa and subserosa of the

duodenal wall. These results may suggest that there may be no presence of free communication between the lymph plexuses of gastric wall and that of the duodenal wall, but they may indicate that when certain disturbances in lymph flow in the wall of pyloric antrum occured, some amounts of lymph in the pyloric region will be discharged through the lymphatic plexuses or spaces to the duodenal wall. Zhdanov has described that the lymph plexuses of gastric wall may freely communicate with that of duodenal wall anatomycally, and that the lymph in the pyloric region physiologically discharged through the lymph plexuses within the gastric wall, but not through that within the duodenal wall, to the peripyloric lymph glands, and the lymph in the starting portion of the duodenum was never discharged through the pyloric wall. However, such lymph flow was easily disturbed on some occasions, for example, in the case of pyloric cancer or of a high pressure caused by the injection of the pigment (21). Therefore, if the cancerous growth exists to some extent in pyloric antrum, it may be possible that cancer cells invade into the duodenal submucosa or subserosa by the lymphatic permiation or by the continuous extension through the lymphatic spaces.

The duodenal spread of the cancer of the cardia and of the body of the stomach was revealed by the presence of cancer cells in the lymphatics within the duodenai subserosa. In these cases, the subpyloric and retropyloric groups of lymph glands were involved by cancerous metastasis and large congromerate of lymph glands was noticed. Anatomically, it is obvious that these lymph nodes receive the lymph flow from the duodenal and pyloric subserosa (17, 20). Therefore, if these glands were involved by cancer cells in the cancer cases of the cardia and of the body, the duodenal invasions may happen owing to the lymphatic permiation of cancer cells. Thus certain disturbances in lymph flow in the gastric wall may play an important role in the duodenal spread of gastric cancer.

Zinninger *et al.* have described that about 30 per cent of the cases of pyloric cancer, which were removed surgically, showed cancerous invasion into the duodenum. Fodden has noticed that the duodenal spread of pyloric cancer was more widespread and destructive in autopsy cases than in surgical cases. The present studies indicated that the duodenal spread of cancer cells occured not only in cases of pyloric cancer but also in cases of the cancer of the cardia of the stomach. Even though cancerous involvements of the duodenum were not recognized grossly, the extent of such invasions varied from a few millimeters to several centimeters. More attention should be paid to the duodenal spread of gastric cancer which will influence the prognosis of cancer patient.

Summary

Twenty-seven cases of gastric cancer were studied with respect to frequency of

spread into the duodenum and mode of invasion. In supplementary experiments through rabbits, the extension of Indian ink injected into the gastric wall to the duodenum was presented.

1) The duodenal spread of cancer cells was noticed in 5 out of 12 cases of pyloric cancer, and in 4 of the 5 the spread was recognized grossly. In 2 out of 5 cases of cancer of the body, in 2 out of 3 cases of the cancer of the cardia, and in 1 out of 2 cases of the diffuse cancer, microscopic invasions of cancer cells into the duodenum were noticed. In those cases peripyloric lymph glands were enlarged and congromerated by the metastatic involvements of cancer. In 3 out of 5 cases of gastric cancer with gastrectomy, the oral stump of the duodenum was microscopically invaded with cancerous lesions. Macroscopic types of those gastric cancers were ulcerative or diffusely infiltrative. Histological types of the gastric cancers had little relation to the frequency of the duodenal spread of the cancer. Those invasions of cancer cells were chiefly noticed in the lymphatics within the duodenal wall, particulary within the subserosa or submucosa.

2) On the experiments on rabbits, in which fairly large amount of Indian ink was injected into the gastric wall near the pyloric ring it reached the subserosa or submucosa of the duodenum for about 1 cm distal from the pyloric ring, but when small amount of Indian ink was injected in the similar portion its extension into the duodenal wall was not recognized. Therefore it is of interest that the physiological lymph flow in the pyloric region may be easily disturbed by a trifling factor.

The above results may suggest that certain disturbances in lymph flows in gastric wall play an important role in the duodenal spread of gastric cancer, and that these spread are chiefly caused by lymphatic permiation of cancer cells or continuous extension of cancer cells through the lymphatic spaces.

The authors are indebted to Prof. Mori, Nagoya City University Medical School, for the helpful advice on lymphatic pathway in stomach.

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Explanation of Plates XXIX—XXXII

Fig. 1 (Case 228) Section of duodenum, showing carcinoma in lymphatic vessels among Brunner's glands. The carcinoma showed the adenocarcinomatous pattern.

Fig. 2 (Case 45) Section of duodenum, showing diffusely infiltrated cancer cells in subserosa and muscularis.

Fig. 3 (Case 148) Section of duodenum, showing carcinoma within the lymphatics in muscle layer.

Fig. 4 (Case 180) Section of duodonum, showing extensive permiation of carcinoma within the periglandular and subglandular lymph plexus.

Fig. 5 (Case 253) Section of duodenum, showing cancerous invasion into subserosa.

Fig. 6 (Case 133) Section of duodenum, showing the mass of cancer within the lymphatics in subserosa.

Fig. 7 (Case 154) Section of duodenum, showing the continuous growth of cancer in subserosa and in muscularis.

Fig. 8 (Case 250) Section of duodenum, showing masses of cancer within the lymphatics in submucosal layer.

Fig. 9 (Case 265) Section of duodenal stump, showing collections of cancer cells in muscle layer.

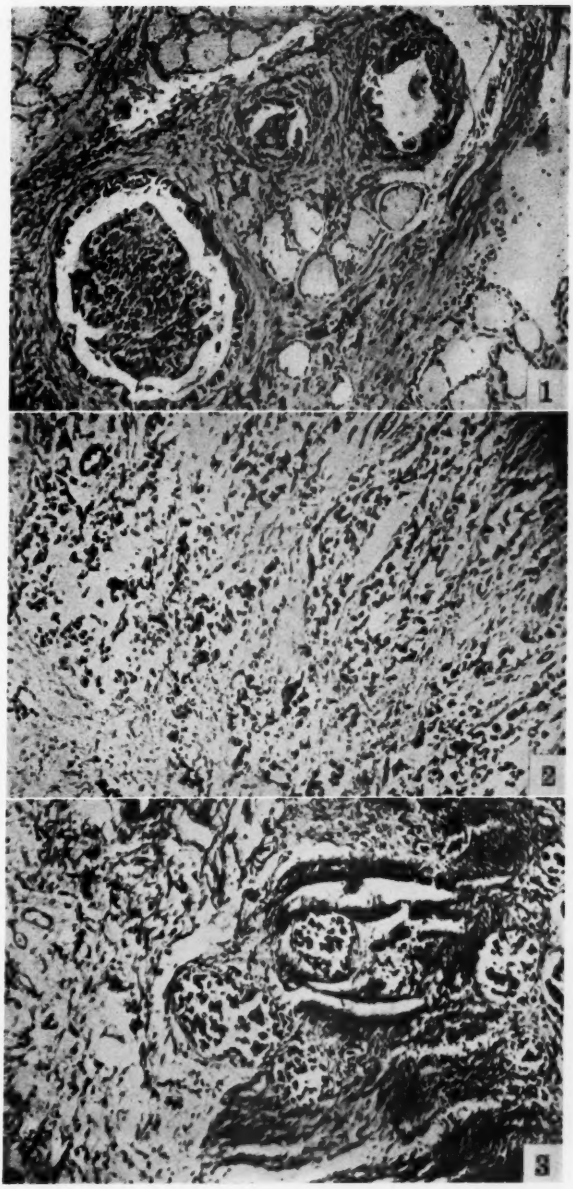
Fig. 10 Transection including stomach of rabbit (Case 3), showing extension of injected Indian Ink into duodenal submucosa from stomach wall.

Allow: (1) Approximate positin of pyloric ring. (2) Limit of extension of Indian Ink into duodenum.

Fig. 11 Transection of the same portion as the above, showing extension of Indian Ink into subserosa of duodenum.

(1), (2) same portion as above.

Fig. 12 Photomicrograph of area 2 in Fig. 10, showing macrophages storing carbon particles between Brunner's glands and muscularis.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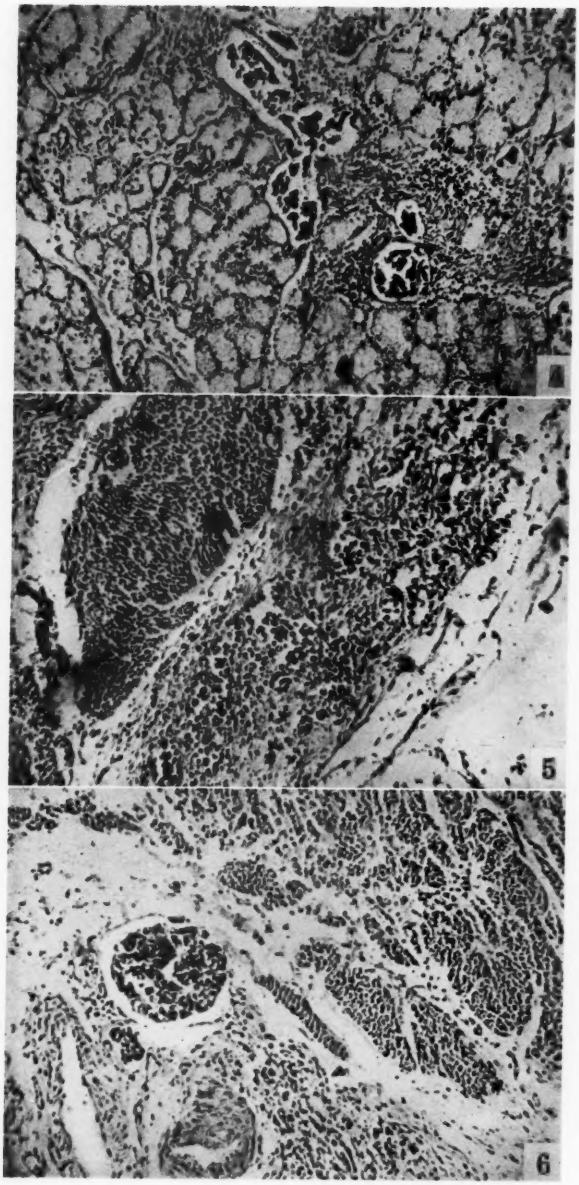
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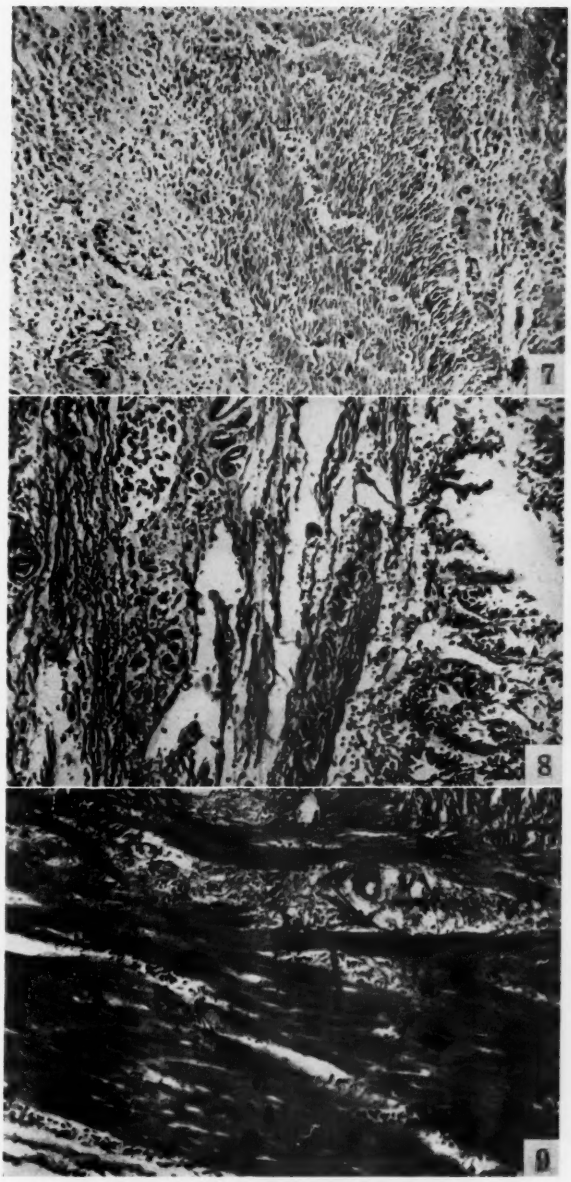
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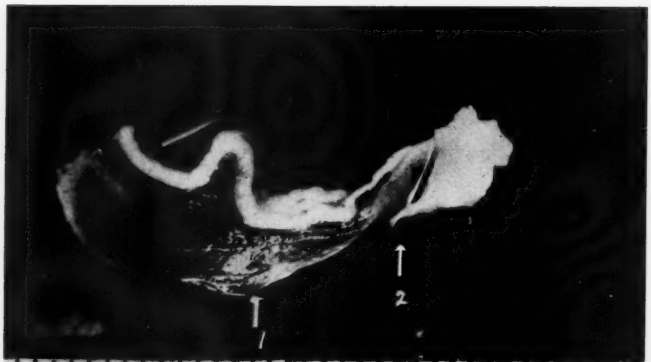

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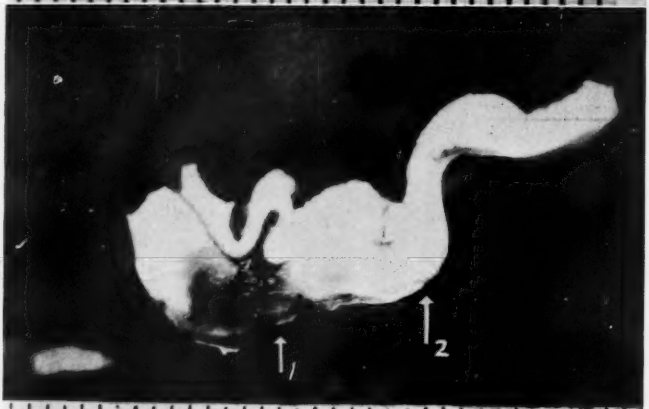

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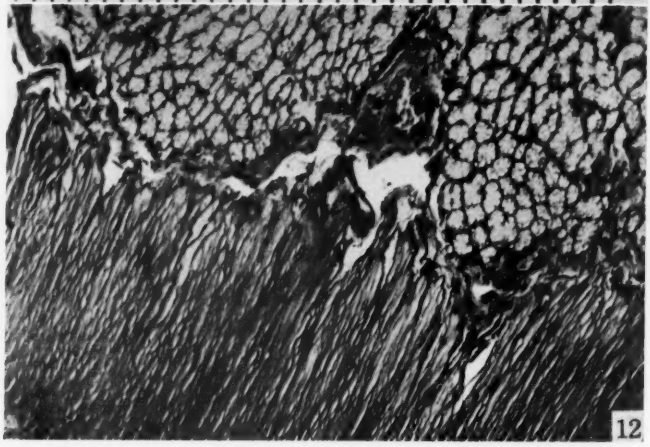

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[GANN, **50**, 417~428; December, 1959]

TRACK AUTORADIOGRAPHIC STUDY ON THE ^{14}C-2-GLYCINE INCORPORATION INTO THE EHRLICH ASCITES CARCINOMA CELLS

(Plates XXXIII and XXXIV)

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INTRODUCTION

The amino acid incorporation into tumor cells in nucleic acid and protein metabolism has been studied by many investigators, most of whom concluded that an enhanced rate of the incorporation can be found in tumor cells in comparison with normal cells; nevertheless no essential difference regarding biochemical composition and finer cytological structure between the tumor and normal cells has been found so far. An exaggerated synthesis of nucleic acid and protein is presumably necessary for tumor cells to attain an extremely high mitotic rate. Zamecnik[37] has reviewed this problem and has interpreted that the incorporation of labelled amino acids into tumors is not necessarily higher than in normal tissues. It does not seem possible, however, that metabolic features in tumor and in normal tissue can be immediately compared since one is under control of harmonious way and the other grows in a malignant way. On the other hand, it has been postulated that a peculiar property of tumor cells to concentrate amino acids was due to the exchange diffusion of amino acids[12),22)].

It must not be forgotten that tumor cells are of two different types which can possibly be distinguished in their protein synthesizing ability as was indicated by Caspersson and Santesson[11)], and by Barigozzi and Dellepiane[5)]. More recently, similar result was also obtained in autoradiographic study with radioactive precursor[13)]. The autoradiographic methods used in this field have developed during the past few years, and provide two methods, one termed "track autoradiography" and the other termed "contrast or grain autoradiography". With either of the two methods it is possible to demonstrate clearly the localization of label within not only tissue but also a single cell in connection with morphology and physiology[6)]. But serious difficulties have been found in these methods; for instance, sensitivity of emulsion, chemical fogging, natural fogging, latent image fading, quantitative availability, etc. However, in the track autoradiographic method, the nuclear research emulsion which is extremely sensitive to radiation particles, especially

for the β-particle, has almost solved these difficulties. It can be definitely recorded even if there is a single β-particle as shown schematically in Chart 1. Recently some attempts have been made in the quantitative application of the method, and a number of extensive studies reported.

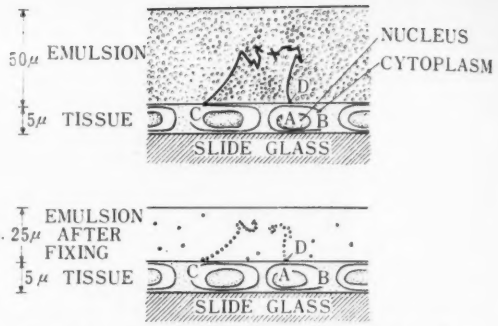


Chart 1. A schematic diagram of a beta track Autoradiograph.
Top: cross section of the emulsion and tissue section before processing. Middle: same as top after processing. Bottom: plan view of interface between emulsion and tissue.

The experiment described in this paper is designed for quantitative evaluation of the incorporation of ^{14}C-2-glycine, following an intraperitoneal injection, into the Ehrlich carcinoma cells with the aid of track autoradiography.

Materials and Methods

The Ehrlich ascites carcinoma cells were propagated in five FJ-2 mice which had received intraperitoneal inoculation of 0.2 ml of ascites cell suspension 5-6 days prior to the experiments. The mice had been previously fed with the ordinary laboratory diet. The ^{14}C-2-glycine, 1500 mc./mole in specific activity, was purchased from the Daiichi Pure Chemicals Co., Ltd., Tokyo. It was dissolved into sterile distilled water at the rate of $2\text{-}2.1\times10^4$ counts/min of the tracer in a volume of 0.1 ml. The each animal was injected intraperitoneally with a single dose of the tracer solution, 0.1 ml/g body weight corresponding approximately to 0.1 μc./g body weight. At an appropriate interval, from 30 minutes to 72 hours after injection, the tumor cells were withdrawn from the peritoneal cavity of the mice and were smeared on several slides each time. These slides were fixed immediately with absolute ethanol. Some of them were used for autoradiographic study, and the rest were used for ordinary microscopical observation after Giemsa staining.

Track Autoradiographic Procedure: This is shown in Chart 2. The autoradiographic procedure used in this study was carried out by the stripping method. The nuclear research emulsions used in this study were "Fuji ET-6 B" type, 50 microns in thickness, and were supplied by the Research Laboratory, Fuji Photo Co., Ltd., Kanagawa. The fixed specimen was dipped into 0.5 per cent of gelatine solution and was dried at room temperature, thus coating it with a very thin gelatine layer. Subsequent handling was carried out in a dark room with the

photographic safe light of Fuji No. 3 glass until the end of the photographic processes. The emulsion was stripped off from its glass support under water, and with it the specimen was covered. After it was dried carefully at room temperature using an electric fan and was placed in a light-tight box with mild desiccant, such as Silicagell grains. The exposure of autoradiograph at 4°C lasted 24 hours. The autoradiograph was developed with diluted FD-111 Developer at 16°C for 3 minutes, and was then fixed in an acid fixer, such as "Fujifix", at 16°C for 20 minutes. It was then washed for 30 minutes under tap-water and was dried at room temperature. After modified Giemsa staining[19], it was dried again at room temperature and was cleared with xylol, and finally mounted in Canada balsam.

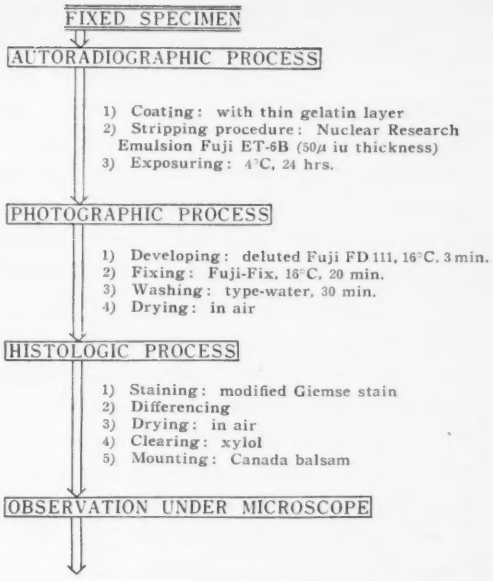


Chart 2. Track autoradiographic procedure.

Observation: The autoradiograph was observed under a light microscope, 500 in magnification. In the emulsion the β-particle makes a track having various figures going any direction. Although it is impossible to observe the whole figure of a track in the same focus, it is possible to do so by slightly changing the focus.

Quantitative data which were corrected statistically after several different countings have been expressed by the track numbers to be counted in 100 tumor cells. According to the site of label, at the same time, the ratio between the counts of track emerged from the nuclei and cytoplasmic areas was calculated. However, the tracks showing ambiguous origin, estimated approximately at 10 per cent or less of the sum, were excluded from the data. At the same time, Giemsa-stained specimens were observed.

Experimental Results

(1) Morphological Findings

It has been well known that the morphological changes of the ascites occur following the inoculation of the Ehrlich ascites carcinoma of a mouse. A little

variety in cellular components of the ascites, especially in quantity of non-malignant cell fraction, has been usually observed in various periods following the tumor inoculation. During 2–3 days after inoculation, it was remarked that there was a large quantity of monocytes, small quantities of lymphocytes, neutrophiles and mast cells, whereas the growth of tumor cells remained almost the same. In a subsequent period, that is in 4–5 days, a rapid increase of tumor cells including a great number of mitotic figures was observed. The volume of ascites increased considerably in the following period. The animals usually died between 8 and 15 days after inoculation. Besides, a small portion of macrophages in the ascites was usually observed, but it did not show constant response. In the later period of tumor growth, the ascites became sometimes hemorrhagic. In a period after 7 days, some tumor cells revealed a degenerative process, such as large cytoplasmic vacuole formation, multinuclear giant cell, etc. Meanwhile it was a peculiar finding that the tumor cells had shown a tendency of cytoplasmic blister formation as seen in Figs., which might be presumed as an artifact occurred during the morphological preparation. That has been interpreted as due to the weakness of the cell membrane. On the other hand, this tendency has hardly been observed in any other sort of ascites tumor cells.

By a radioactive glycine injection, no remarkable changes except a slight decrease in the number of dividing cells and an occasional apparent increase of macrophages, were observed.

(2) Track Autoradiographic Results

(*a*) ***Resting tumor cell*** (***Interphase***): In time, a quantitative evaluation followed after the glycine injection had been made as shown in Table 1. According to these values, the time-courses regarding the distribution of radioactivity have been drawn in Chart 3. At the same time, regarding the localization of label, a ratio

Table 1. The track numbers in 100 tumor cells and the ratio between the track numbers originated in nuclei and those in cytoplasms with time after ^{14}C-glycine injection.

Time / Mouse	30 min. (N/C)	1 hr. (N/C)	2 hrs. (N/C)	4 hrs. (N/C)	6 hrs. (N/C)	12 hrs. (N/C)	24 hrs. (N/C)	48 hrs. (N/C)	72 hrs. (N/C)
No. 1	102.5 (1.61)	97.7 (2.54)	60.2 (2.28)	55.2 (3.01)	—	37.2 (3.28)	20.8 (5.51)	15.2 (7.33)	—
No. 2	101.0 (1.64)	91.0 (2.37)	69.8 (2.48)	69.0 (2.19)	—	42.3 (2.41)	29.8 (2.71)	23.0 (4.86)	10.2
No. 3	—	96.2 (2.11)	—	42.0 (3.26)	—	27.7 (3.22)	26.2 (4.83)	—	—
No. 4	—	119.8 (2.07)	76.4 (2.81)	46.6 (4.11)	20.0 (3.89)	16.8 (4.20)	16.8 (4.12)	—	7.8
No. 5	—	111.2 (3.21)	81.8 (2.93)	29.8 (3.03)	17.3 (4.81)	13.0 (5.03)	12.9 (7.02)	—	6.5

(N/C): N; track numbers originated in nuclei
C; track numbers originated in cytoplasms

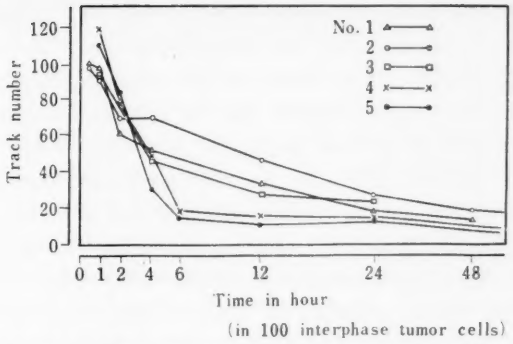
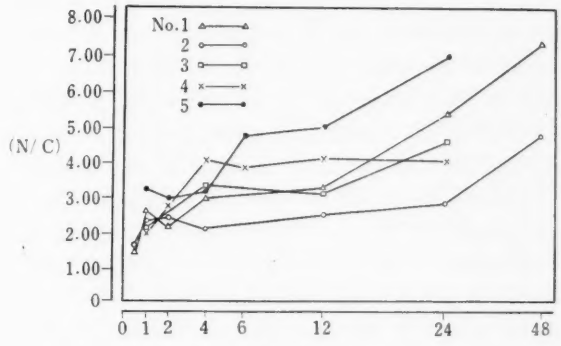


Chart 3. The time-course of ^{14}C-glycine incorporation into the tumor cells.



$$N/C : \frac{\text{track numbers originated in nuclei}}{\text{track numbers originated in cytoplasms}}$$

(in 100 interphase tumor cells)

Chart 4. The time-course of the ratio between track numbers originated in nuclei and those in cytoplasms.

between track counts originated in nuclei and those in cytoplasms has been shown in (N/C) of Table 1, with which each of their time-courses were also given in Chart 4.

The uptake of glycine did not distribute equally in all the cells, even if it had been considered as the interphase cells; i. e., a distinct difference regarding the ability of glycine-uptake could be easilly observed in the following two types of cells. Cells having rich chromatin nuclei and a middle sized deep stained cytoplasm showed a more intensive uptake. On the other hand, cells having poor chromatin nuclei and a less stained cytoplasm of similar size, the uptake was considerably smaller. At the localization of label, the uptake in both nucleus and cytoplasm was clearly observed, but the former gained advantage with the lapse

of time. The majority of tracks originated in nuclei, i. e., in nuclear substances, which were closely associated with the nuclear membrane. Nevertheless it is well known that nucleolus plays a very important role in protein metabolism. Although some tracks originated in the nucleoli, but no reliable evidence supporting this fact, has been derived from this study. In fact, the nucleolus in the Ehrlich ascites tumor cell reveals a relatively obscure appearance even under an ordinary microscope. In addition, in the track autoradiograph, the thick nuclear research emulsion laid on the specimen may interfere somewhat in microscopy. Although in the cytoplasmic area, the uptake was steadily observed, the quantity was less compared with that of the nucleus. It was an interesting finding that some of the tracks had originated in the vicinity of the nuclear membrane within a cytoplasmic area. This finding, related with the above mentioned tracks of the nuclear substances, might be suggested as a certain part of transfer mechanism between nucleus and cytoplasm, such as nucleoprotein or its precursor.

Thirty minutes after the injection, the uptake of glycine into the interphase cells was evaluated as the highest throughout the whole period of this study. At 1 hour after the injection, an exaggerated uptake seen at 30 minutes was decreased somewhat. Therefore each track could be clearly and separately distinguished from each other. It was also observed that the uptake in the hyperchromic cells was greater than in those of the hypochromic cells. Labeling in nucleus was also greater than in cytoplasm. This tendency was expressed more clearly with the time lapse. The ratio of N/C was calculated at 1.6. Two hours after the injection, the distribution of glycine showed a continuously rapid decrease in both the nucleus and cytoplsm, however, it seemed rather remarkable in cytoplasm. Four hours after the injection, the decrease of glycine distribution reached about one-thirds of the value obtained after 1 hour. A continuous rapid decrease of radioactivity could be observed in the early period, while after 4 or 6 hours, radioactivity was maintained almost at the same level.

(*b*) ***Dividing tumor cell***: Although in every phase of mitosis, such as prophase, metaphase, anaphase and telophase track originated in their chromosomal substances could be observed as shown in Figs. This fact does not seem to indicate a direct incorporation and synthesis into chromosomal substances during their mitotic period. These synthesis might occur during an interphase as supposed from the evidence that most of the tracks associated with mitotic figure had been usually found in a later period after the glycine injection. Nevertheless, no direct evidence that chromosom duplication occurs during the interphase, as supposed by numerous investigators, was obtained in this experiment. At the moment, further investigation is in progress on this point.

(*c*) ***Miscellaneous findings***: It is notable finding that tumor cells in the degen-

erative process, having huge cytoplasmic vacuole, etc., as shown in Fig., revealed a great number of tracks. These tracks originated mainly in altered nuclei, and they did not originate in vacuole. Moreover, this exaggerated uptake by degenerative cells was also observed even in later periods, i. e., radioactivity of these cells tends to maintain the same level. Some of them, on the contrary, revealed as little an uptake as in the usual way. On the other hand, in the non-malignant cell fraction of the ascites, the uptake of glycine was also observed. Especially, macrophages and free serosa-epithelia showed an exaggerated uptake which was estimated as several times larger than those of the tumor cells. In this case, radioactivity decreased considerably in a later period. The uptake in monocytes, lymphocytes, etc., was estimated to be little in the early period and merely traceable in later periods.

Discussion

Autoradiography: Some attempts have been recently made to use autoradigraphic methods quantitatively: one is the grain counting method reported by Mazia[26], Levi[24], Pelc[30] and Gullberg[21], and the other is the track counting method reported by Levi[24], Ficq[14] and Guidotti[20]. Both of these methods have their proper advantages. Track autoradiography is especially suitable for low energy β-particles, for example, the ^{14}C-glycine used in this study. The average length of their tracks is calculated to be 10–15 microns, therefore the "Fuji ET-6 B" type emulsion with 50 microns of thickness is suitable for the purpose. Nevertheless, the final thickness of the emulsion will be about half the value given at the original state, i. e., if the emulsion on water swells to twice its area in the dry state during the stripping procedure, and then after drying with the specimen it contracts in the vertical dimension only. However, according to the dealer of this emulsion, its composition and sensitivity are evaluated satisfactory[29]. Regarding the localization of label, the individual track under microscope can be traced back exactly to its origin in relation with the cytological structure. Then the microscopical resolution of it was the order of 1–2 microns. Although one of the important factors concerning the accuracy of autoradiographic study depends on the period of time exposure, the period of time exposure applied in this study would be proper.

^{14}C-glycine incorporation: So far no striking differences have been found between the amino acids composition of tumor protein and nucleoprotein, and those obtained from normal tissues as reported in our previous papers[3),4)] and other extended papers[9]. Whereas, by injecting an animal with radioactive glycine and determining the radioactivity of the cell proteins and nucleic acids after different intervals of time, it could be possible to determine the rate of incorpration of

glycine into the proteins and nucleic acids. However, before reliable conclusions can be drawn from these experiments, various important factors must be taken into consideration.

As to the incorporation of the ^{14}C-glycine into the Ehrlich ascites carcinoma cells, the maximal peak was found after the first 30 minutes of injection. This fact agrees undoubtedly with numerous investigations[7)~10),15),25),36),37)]. Indeed, the glycine incorporation into tumor proteins and nucleic acids begins rapidly within only a few seconds. Meanwhile, ascites tumor cells have the ability of concentrating glycine into the cells. According to the extensive studies of Christensen et al[12)], and Heinz[22)], most of the intracellular glycine would be free, and the concentration might be caused by the exchange diffusion; therefore it can be presumed that the rapid decrease of radioactivity observed in the early period can be explained by the participation of this mechanism. Although the distribution of radioactivity in the successive periods was maintained almost at the same level, in this period the intracellular glycine might be considered as a component of proteins and nucleic acids. On the contrary, exchangability of glycine between tumor cells and extracellular fluid was steadily observed as mentioned above. Thus the one-way passage of glycine could not be observed. In relation to cell structure and glycine incorporation, there were two different types of tumor cells regarding the ability of incorporation that the hyperchromic cells showed larger uptake than the hypochromic cells. This fact agrees with the autoradiographic study of DePaepe and Ficq[13)] using the human uterine cancer tissue with the ^{14}C-8-adenine and ^{14}C-2-phenylalanine. Moreover, there is distinct evidence that the majority of tracks originate in the nuclear substances, which may be mostly DNA. In fact, one of the interesting results in this experiment is the localization of label in the vicinity of the nuclear membrane. The track in the cytoplasmic area was also localized in the outer vicinity of the nuclear membrane in many cases. These findings suggested that in protein and nucleic acid synthesis the nuclear membrane might play a certain important role as to the interaction between nucleus and cytoplasm. This is also supposed by the electrone microscopical study[2)] indicating that elgastoplasm may be derived from the nuclear membrane. Furthermore, in the autoradiographic study of Goldstein[17),18)], it was clearly shown that the nucleoprotein may be transferred from the nucleus to cytoplasm, and it is also suggested that the nuclear membrane may be freely permeable to molecules which are as large as proteins. Besides, in the biochemical study of Osawa et al.[1),28)] they obtained the dominant activity of nucleus on the incorporation of glycine into protein. On the contrary, in the autoradiographic study on the ^{35}S-methionine uptake into tumor cells, Mutolo et al.[27)] concluded that protein synthesis occurs in cytoplasmic territory. This finding differs definitely from the results

obtained in the present study respecting the localization of label; however, both might be correct. According to the data of Allfrey et al[1] the incorporation of ^{35}S-methionine into protein is several times as large as that of ^{14}C-glycine. Tyner et al.[35] studied the ^{14}C-glycine incorporation into protein and nucleic acids purines, such as RNP, DNA, using Flexner-Jobling carcinoma, and showed about a tenfold greater specific activity of nucleic acids purines compared to protein. Therefore, if ^{14}C-glycine is used in an experiment with tumor, result will show mainly the incorporation into nucleic acids, in particular DNA. In addition, Busch and Greene[8] demonstrated that the plasma protein labelled with ^{14}C-glycine could be easily utilized into the tissue proteins. This is also one of the reasons for the above mentioned difference, i. e., intraperitoneal and intravenous injections of isotopes. Allfrey et al.[1] thus concluded in their studies of isolated cell nuclei, that protein synthesis occurs in both nucleus and cytoplasm. From the present experiment, it is still obscure whether protein synthesis may occur only in nucleus or both in nucleus and cytoplasm. One of the peculiarities of malignant cells might be that most of the incorporation can take place in the nuclear substances, such as DNA or the precursor, in close relation with the nuclear membrane. Numerous investigators[7),32] concluded that the nucleolus showed the highest activity in the synthetic process, but controversy is still present on this problem. No striking evidence of the increased activity of nucleoli was obtained from this experiment.

Meanwhile, in the dividing tumor cells, the tracks originating in certain parts of the chromosomal substances were obviously observed in each different stage of mitosis, indicating that the glycine has been incorporated into the chromosomal substances. Unfortunately, no conclusion about their synthetic specifity in malignant cells can be drawn at this moment. It has been indicated that chromosomal duplication in normal cells occurs during the interphase of pre-mitotic phase.[16),31),33),34] As shown in this experiment, mitotic cells could reveal some tracks but their radioactivity was definitely less than those of the interphase. According to Howard and Hornsey's study[23] using the ^{14}C-8-adenine, the time interval of mitotic cycle of the Ehrlich ascites carcinoma cells may be estimated to be 15–18 hours, and the chromosomal duplication to occur in pre-mitotic phase. From the present experiment, it is strongly supposed that the synthesis of chromosomal substances takes place in the period before prophase under the existence of the nuclear membrane. On the other hand, an enhanced glycine uptake was observed in the degenerative tumor cells, macrophages, and serosa-epithelia. It is perhaps ascribable to their altered nuclear membrane, which could not act in the normal way. Therefore, the incorporated glycine may hardly be regarded as a metabolic substitute.

Summary

1) Glycine incorporation into the Ehrlich ascites carcinoma cells was studied quantitatively after ^{14}C-2-glycine injection with the aid of track autoradiography.

2) The autoradiographic method was discussed, especially the quantitative track autoradiography.

3) Glycine incorporation into the interphase tumor cells was rapid, its maximal peak occurring at least 30 minutes after injection.

4) As to the distribution of radioactivity, a rapid decrease in the early period and almost the same level in subsequent period were observed. The former was presumably ascribable to the exchangability of glycine and the latter to the behavior of glycine incorporated into nucleic acid, protein or the precursor.

5) Glycine incorporation into tumor cells, i. e., into their nucleic acid, protein or precursor, took place mostly in the nucleus, and the nuclear membrane played a certain important role, but it may occur also in the cytoplasm in part.

6) Two different types of tumor cells could be observed for their ability of incorporation. Cells having rich chromatin nucleus and deep stained cytoplasm showed an intensive uptake, while cells having poor chromatin nucleus and less stained cytoplasm were inactive.

7) Considerable quantities of the tracks could be observed in the degenerative tumor cells and some of the non-malignant cell fractions.

8) The dividing tumor cells showing uptake were observed and related problem was discussed.

Acknowledgments

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Explanation of Plates XXXIII and XXXIV

a) Photographed at the cell level.
b) Photographed at the emulsion layer above the cell.

Figs. 1–8. Interphase tumor cells.

Fig. 1. One hour after the injection, showing two tracks originated at the center of nucleus.

Fig. 2. One hr., Showing a track originated in nucleus, in which it was in the vicinity of nuclear membrane.

Fig. 3. Four hrs., above cell: showing a track originated in cytoplasm. The other two cells are showing a track originated in nucleus respectively.

Fig. 4. Twelve hrs., showing a track orginated in cytoplasm, in particular in the vicinity of nuclear membrane.

Fig. 5. One hr., showing atrack in each cell. above cell : it originated in cytoplasm. middle cell : it originated in nucleus. bottom cell : it originated in nucleus.

Fig. 6. Twenty-four hrs., showing a track originated in nucleus, in particular in the vicinity of nuclear membrane.

Fig. 7. Four hrs., showing two tracks originated in nucleus, one is at center, the other is in the vicinity of nuclear membrane.

Fig. 8. Twelve hrs., showing three tracks originated in cytoplasm, one is in the vicinity of nuclear membrane.

Figs. 9-13. Dividing tumor cells.

Fig. 9. Four hrs., prophase ; showing two tracks, one of them originated in chromosome.

Fig. 10. Twenty-four hrs., metaphase, showing a track originated in cytoplasmic area.

Fig. 11. Twelve hrs., anaphase, showing a track originated in one of chromosomal mass, had been divided into two parts.

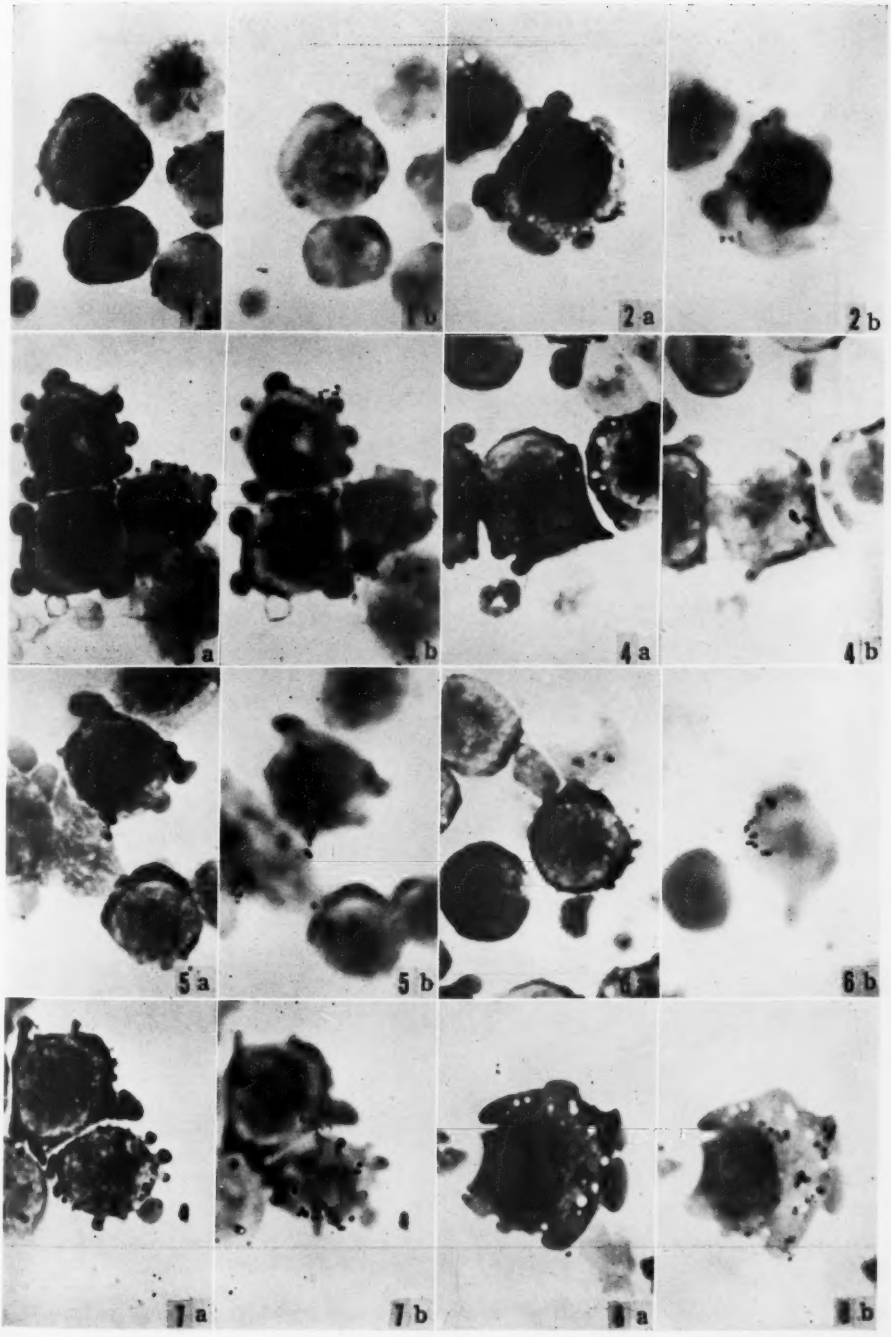
Fig. 12. Twenty-four hrs., same as Fig. 11.

Fig. 13. Twelve hrs., telophase; showing a track originated in one of divided chromosomal mass.

Fig. 14. Four hrs., a degenerative tumor cell is showing numerous tracks, which originated mostly in the altered nucleus.

Fig. 15. Twelve hrs., a degenerative tumor cell is showing numerous tracks, while a interphase tumor cell (bottom) is showing only two tracks originated in nucleus.

Fig. 16. One hr., non-malignant cells, a macrophage (large sized), two serosa-epithelium cells (middle sized) and a monocyte (small sized) are seen, and are showing numerous tracks except monocyte.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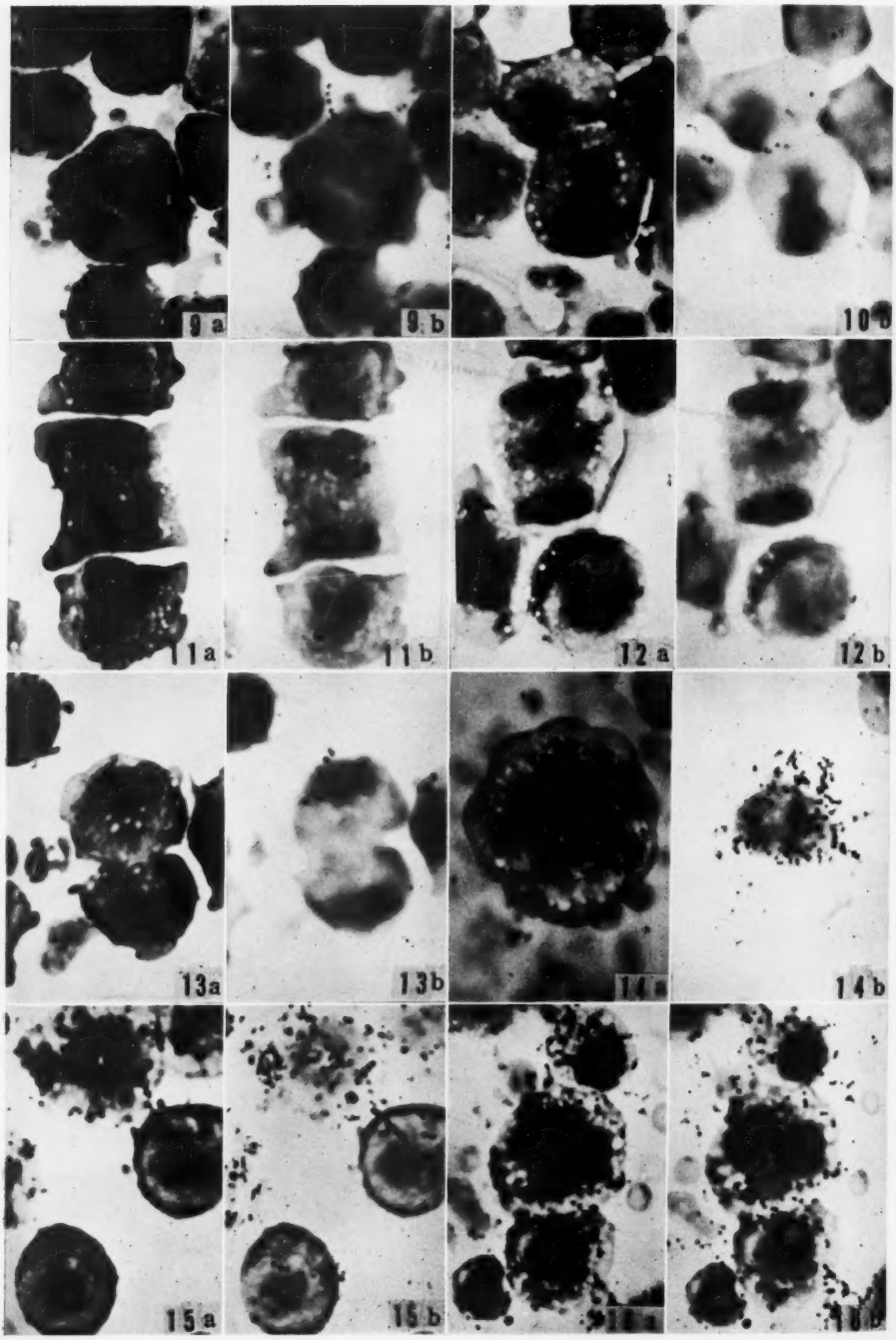
9a
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[GANN, **50**, 429～436 ; December, 1959]

ISOLATION OF THE MITOSIS PROMOTING SUBSTANCE, ONCOTREPHIN, FROM RAT ASCITES HEPATOMA (AH 130)*

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INTRODUCTION

In the previous communication[6] the authors reported that a substance (or substances) can be isolated from certain rapidly growing tumors of man. This substance promotes mitosis of the epidermis of mouse ear *in vitro*, and was named "oncotrephin". In another communication[7] they have also demonstrated that the same substance promotes the growth of strain L cells in tissue culture. Further studies on the nature of oncotrephin have been made and are still in progress. In this paper the results are given of experiments demonstrating the existence of oncotrephin in an experimental hepatoma (AH 130, kindly supplied by Dr. Katsuta) of rat.

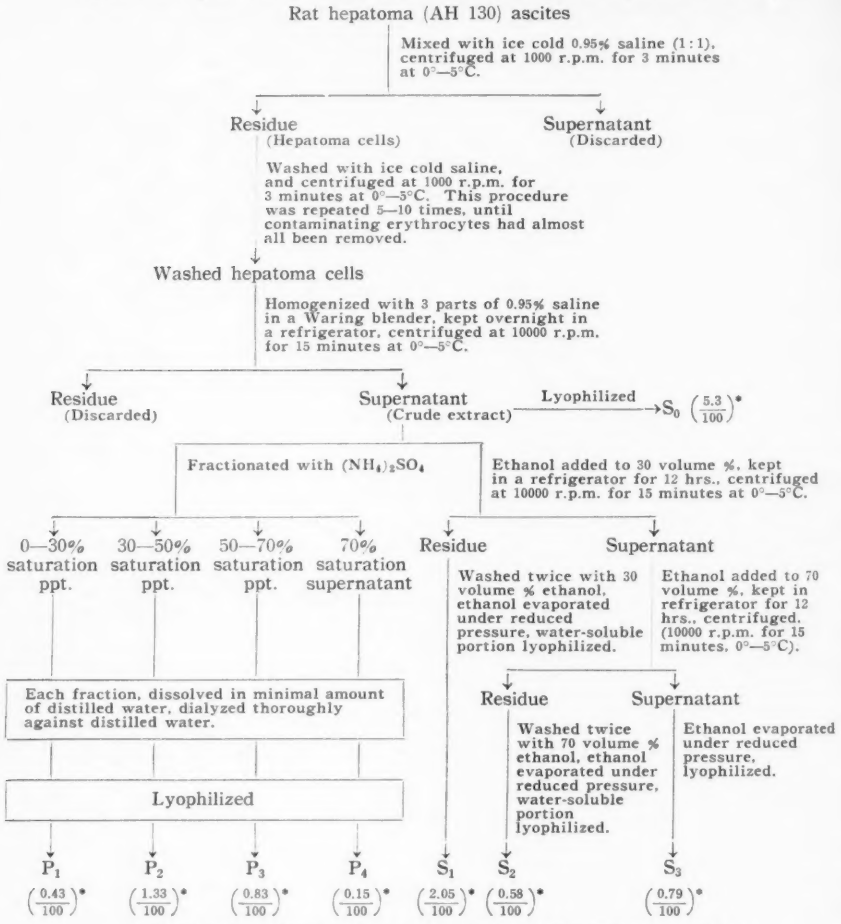
MATERIALS AND METHODS

1. *Isolation of oncotrephin from ascites hepatoma (AH 130) cells of rat.* A half ml of rat hepatoma ascites was implanted into the peritoneal cavity of hybrid rat. On the 8 th～10 th day after transplantation, the animals were sacrificed and the ascites obtained were pooled. Hepatoma cells in the ascites were washed with ice cold saline solution until they were almost free of contaminating erythrocytes. Saline solution was added to the washed cancer cells and they were homogenized in a Waring blender. After standing overnight in the refrigerator the homogenate was centrifuged at 10,000 r. p. m. for 15 minutes at 0°～5°C. Then, the supernatant, crude extarct, was fractionated with ethanol as described in our previous report.[6] Furthermore, four fractions were obtained from the crude extract by $(NH_4)_2SO_4$ precipitation. They were dialyzed for 24～48 hours against distilled water until the dialysate gave no Nessler reaction. They were then lyophilized.

The procedure used is shown in Table 1. The yield of each fraction, expressed as the dry weight, is presented in this table per wet weight of the washed cells. Fractions were dissolved in distilled water and their activity was assayed. The

* A part of the research expenses was paid by the Scientific Research Fund of the Ministry of Education of Japan.

Table 1. Preparation of "Oncotrephin" from rat ascites hepatoma (AH 130).



* Numbers in parentheses indicate yield of fraction designated in dry weight per wet weight washed cancer cells.

nitrogen content of the solution to be tested was determined by the micro-Kjeldahl method (modified by Parnas).

2. ***Evaluation of mitosis promoting activity of individual fractions of rat hepatoma on the epidermal cells of mice pinna in vitro.*** Bullough's method[1),2)] was used as described in the previous report.[6)] Ear fragments of adult C3H/HeN (kindly supplied by Prof. H. Satô) male mice aged from 3 to 6 months were incubated for one hour in a culture solution containing glucose. Then the solution of test material in distilled water and colchicine solution were added and the incubation was continued for four hours. The number of mitoses in the Malpighian layer, arrested

Table 2. Effect of fraction S_0, S_1, S_2 and S_3 prepared from rat ascites hepatoma (AH 130) on the mitosis of epidermal cells of mouse pinna.

The average number of mitoses arrested by colchicine in unit length (1 cm) of 7 μ thick sections of mouse ear epidermis, incubated for 4 hrs. at 38°C in the culture medium. In a), b), c), d) are shown the results with S_0, S_1, S_2, S_3 respectively.

a)

Number of ear fragment	Frequency of mitoses per unit length	
	Control	S_0 0.031*
1	0.8	0.9
2	0.8	7.2
3	1.1	5.1
4	1.4	4.9
5	3.2	7.9
6	4.3	8.7
7	7.3	0.8
Average	2.7	5.1

b)

Number of ear fragment	Frequency of mitoses per unit length	
	Control	S_1 0.018*
1	2.9	3.2
2	4.5	2.3
3	7.2	8.9
4	7.3	20.6
5	10.9	9.7
6	10.9	29.3
7	17.2	28.5
8	18.5	21.7
9	19.8	20.9
10	21.8	5.7
Average	12.1	15.1

c)

Number of ear fragment	Frequency of mitoses per unit length	
	Control	S_2 0.024*
1	0.8	3.6
2	1.6	3.3
3	1.6	14.6
4	1.7	5.7
5	2.4	11.8
6	3.8	9.0
7	4.4	9.0
8	4.8	6.8
9	4.9	10.4
10	5.0	3.5
11	5.0	8.8
12	6.0	13.3
13	7.1	2.0
14	7.2	7.7
15	9.8	16.2
16	12.4	7.8
17	12.7	4.6
Average	5.4	8.1**

d)

Number of ear fragment	Frequency of mitoses per unit length	
	Control	S_3 0.036*
1	4.8	12.9
2	6.7	7.3
3	8.3	8.2
4	9.9	8.8
5	10.4	13.9
6	14.0	17.0
7	14.3	14.8
8	15.8	16.4
9	17.9	8.9
10	22.0	13.2
Average	12.4	12.1

* Final concentration designated by mg of nitrogen per ml.

** The difference from the control is statistically significant ($p<0.05$).

at metaphase, was counted in a 10 cm length of stained epidermis. The number of mitoses per 1 cm epidermis of ear fragments cultured in a medium containing the test material was compared with that of a control incubated without the test material.

Results

1. *Mitosis promoting effects of fractions prepared by ethanol precipitation.* At a concentration of 0.031 mg (nitrogen) per ml of culture medium, fraction S_0, crude extract of rat ascites hepatoma cells, apparently increased mitosis of epidermal cells (Table 2). However, the result is statistically insignificant ($p<0.2$).

Table 3. Effect of fractions P_1, P_2, P_3 and P_4 prepared from rat ascites hepatoma (AH 130) on the mitosis of epidermal cells of mouse pinna.

The average number of mitoses arrested by cholchicine in unit length (1 cm) of 7 μ thick sections of mouse ear epidermis incubated for 4 hrs. at 38°C in the culture medium. In a), b), c), d) are shown the results with P_1, P_2, P_3, P_4 respectively.

a)

Number of ear fragment	Frequency of mitoses per unit length	
	Control	P_1 0.015*
1	0.8	2.1
2	3.0	6.5
3	5.1	2.3
4	5.9	8.5
5	7.9	4.5
6	8.3	3.2
7	11.8	2.8
Average	6.1	4.2

b)

Number of ear fragment	Frequency of mitoses per unit length	
	Control	P_2 0.014*
1	0.3	1.7
2	0.6	3.4
3	1.1	6.6
4	4.8	2.9
5	5.9	4.7
6	5.9	9.4
7	8.8	0
8	9.7	20.2
Average	4.6	6.1

c)

Number of ear fragment	Frequency of mitoses per unit length	
	Control	P_3 0.015*
1	0.7	15.7
2	2.2	5.4
3	2.5	19.8
4	5.0	1.6
5	7.1	22.9
6	8.7	18.5
7	8.8	4.2
Average	5.0	12.6**

d)

Number of ear fragment	Frequency of mitoses per unit length	
	Control	P_4 0.015*
1	1.6	4.8
2	2.4	6.6
3	4.0	12.8
4	6.3	8.1
5	7.5	8.6
6	8.1	2.6
7	12.3	15.1
Average	6.0	8.3

* Final concentration designated by mg of nitrogen per ml.

** Difference between P_3 and control is statistically significant ($p<0.1$).

At a concentration of 0.024 mg (nitrogen) per ml, fraction S_2, precipitated by 30 ~70% ethanol from crude extract, considerably increased mitosis (statistically significant; $p<0.05$). Fractions S_1 and S_3 had no effect at similar concentrations (Table 2).

2. *Mitosis promoting effects of fractions prepared by $(NH_4)_2SO_4$ precipitation.* Fraction P_1 at a concentration of 0.015 mg (nitrogen) per ml slightly inhibited mitosis (Table 3). Fractions P_2 and P_4 at similar concentrations slightly increased mitosis. Their effects were insignificant statistically. Fraction P_3, precipitated by 50~70% saturation of $(NH_4)_2SO_4$, had a remarkable stimulatory effect (statistically significant at $p<0.1$) at a concentration of 0.015 mg (nitrogen) per ml.

3. *Mitosis promoting effects of fraction S_2–P_3.* Fraction S_2 was refractionated with $(NH_4)_2SO_4$. The activities of fractions S_2–P_1, S_2–P_2 and S_2–P_4 could not be tested, because of their low yield. Fraction S_2–P_3, at a concentration of 0.031 mg (nitrogen) per ml, had a stimulatory effect (statistically insignificant; $p<0.25$) (Table 4).

Table 4. Effect of fraction S_2–P_3 prepared from rat ascites hepatoma (AH 130) on the mitosis of epidermal cells of mouse pinna.

The average number of mitoses arrested by colchicine in unit length (1 cm) of 7 μ thick sections of mouse ear epidermis, incubated for 4 hrs. at 38°C in the culture medium.

Number of ear fragment	Frequency of mitoses per unit length	
	Control	S_2–P_3*
1	0.6	8.4
2	1.9	7.0
3	2.4	4.0
4	2.8	7.0
5	3.7	4.8
6	4.4	6.3
7	6.1	11.0
8	6.2	6.2
9	6.6	1.3
10	7.8	16.5
11	11.4	1.0
12	15.0	18.5
Average	5.7	7.6**

* Final concentration of fraction S_2–P_3 in culture medium was 0.031 mg nitrogen per ml.

** Difference between S_2–P_3 and control is statistically insignificant ($p<0.25$).

Table 5. Effect of fraction S_2 prepared from normal rat liver on the mitosis of epidermal cells of mouse pinna.

The average number of mitoses arrested by colchicine in unit length (1 cm) of 7 μ thick sections of mouse ear epidermis, incubated for 4 hrs. at 38°C in the culture medium.

Number of ear fragment	Frequency of mitoses per unit length	
	Control	Rat liver S_2*
1	1.1	14.7
2	4.4	27.1
3	5.0	12.3
4	7.2	3.4
5	8.5	5.2
6	10.8	11.9
7	13.4	12.1
8	16.5	11.4
9	18.2	20.2
10	20.0	3.8
11	1.4	3.8
12	4.0	1.4
13	4.6	6.3
14	5.8	2.1
15	7.5	2.0
Average	8.6	9.2

* The final concentration of fraction S_2 in culture medium was 0.047 mg nitrogen per ml.

4. *Effect of fraction S_2 prepared from normal rat liver.* Liver was excised from normal hybrid rats and perfused with cold saline to remove blood cells. It was minced with saline solution and the crude extract was fractionated with ethanol as described in Table 1.

The effect of fraction S_2 from normal liver, precipitated by 30~70% ethanol, was tested. At a concentration of 0.047 mg (nitrogen) per ml no stimulatory effect was observed (Table 5).

Discussion

Oncotrephin, the mitosis promoting substance, which we[6),7)] isolated from certain rapidly growing tumors of man is distinguishable in two respects from other growth-promoting substances hitherto reported. Firstly, it considerably stimulated mitosis of normal mouse epidermis *in vitro*. Secondly, it seems not to be limited to specific tumors, for a substance with a similar effect could be isolated not only from human testicular tumors but also from human sarcomas. The present studies were carried out to see if this substance is a characteristic component of malignant tumors. The ascites hepatoma (AH 130) of rat was chosen for this work and the crude extract was fractionated by precipitation with different concentrations of ethanol. As in the previous studies with human material, the fraction precipitated by 30~70% ethanol was effective on mouse epidermis. Since the isolation of the effective substance, oncotrephin, from experimental malignant tumors became possible, a step was made in its purification. The effective substance is not only precipitated by 30~70% ethanol but also by 50~70% saturation of ammonium sulfate. As the ammonium sulfate precipitate was effective after dialysis, the whole or at least a considerable portion of oncotrephin is presumably non-dialysable.

Though the hepatoma used in the present experiment is highly anaplastic, it originally developed in a rat liver. Therefore, the identification of the effective substances with the growth-promoting agents hitherto isolated from non-cancerous liver may be necessary.

After partial hepatectomy some substances seems to be liberated from regenerating liver, which promotes mitosis in the liver. Friedrich-Freksa and Zaki[5)] (1954) stated that the intravenous injection of partially hepatectomized rat serum stimulated mitosis in normal rat liver. Paschkis, Cantarow and Goddard[11),13)] (1957) have demonstrated that in partially hepatectomized rat an increase in the mitotic rate can be observed in the liver and the cornea, but not in the skin (pinna). Paschkis, Cantarow, Stasney and Hobbs[10)] (1955) found moreover that in partially hepatectomized rats the growth of transplanted Walker 256 tumor is enhanced.

Previous to these investigations McJunkin and Breuhaus[8)] (1931), Wilson and

Leduc[15] (1947) and Teir and Ravanti[14] (1953) showed that if injected intraperitoneally into rats, homogenized rat liver causes an increase of mitosis in the liver. Paschkis and his collaborators[13] prepared an aqueous extract from a rat liver homogenate and injected it subcutaneouly into rats. They showed not only a stimulation of mitosis in liver and cornea (though not in the skin; Paschkis, Cantarow and Goddard,[11] 1957), but also an enhancement in the growth of a transplanted Walker 256 tumor (Cantarow, Paschkis and Stasney,[3] 1955). An analogous effect of a liver homogenate upon a transplanted Novikoff hepatoma was reported by Dufour and Martel[4] (1957). According to Martel, Dufour and Allard[9] (1957), the effective substance is present in the sediment when the homogenate is rapidly centrifuged. Moreover, Paschkis *et al.*[12] (1958) have demonstrated that the effective substances are heat-labile. They also found that after dialysis for 24 hours either the dialysate or the residue alone are ineffective, whereas on recombination the activity reappears.

It can be said therefore that the effective substance in fraction S_2 of rat ascites hepatoma differs from the above mentioned growth-promoting agents in the liver.

Summary

Oncotrephin was isolated from experimental hepatoma (AH 130) of rat. It can be precipitated not only by 30~70% ethanol, but also by 50~70% saturated ammonium sulfate. The question of the identity of oncotrephin with the growth-promoting factors in the liver (Teir *et al.* Paschkis *et al.* and Martel *et al.*) was discussed.

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Errata

Gann, Vol. 50 (1959) page 118, footnote

for	*τρόφειν*
read	*τρέφειν*

[GANN, **50**, 437~444; December, 1959]

EFFECT OF ONCOTREPHIN, THE MITOSIS PROMOTING SUBSTANCE, ISOLATED FROM RAT ASCITES HEPATOMA (AH 130) ON PROLIFERATION OF STRAIN L CELLS*

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Introduction

Oncotrephin[5] is a substance which promotes the mitosis of the epidermal cells of mice pinna *in vitro*. It was originally isolated from certain rapidly growing malignant tumors of man. This substance promotes the growth of strain L cells,[6] if added at optimal concentration to the culture medium. Since the existence of oncotrephin could be demonstrated in the same fraction of rat ascites hepatoma[7], its effect upon the propagation of strain L cells was tested, and its characteristics were examined.

Materials and Methods

1. *Isolation of oncotrephin from ascites hepatoma (AH 130) cells of rat.* The method for isolation of oncotrephin is the same as that described in the previous reports,[5],[7] the whole procedure is indicated in Table 1.

2. *Evaluation of growth promoting activity of individual fractions of rat hepatoma in tissue culture of strain L cells.* A simplified replicate tissue culture method was used.[3],[4],[6] YLH medium containing 5% bovine serum was used as the culture medium. In the experimental groups, the test materials were each added to the culture medium while no test material was added to the control. Cultivation was continued for a week, and on the 2nd, 4th and 7th days the number of cell nuclei per tube was counted in a hemocytometer.

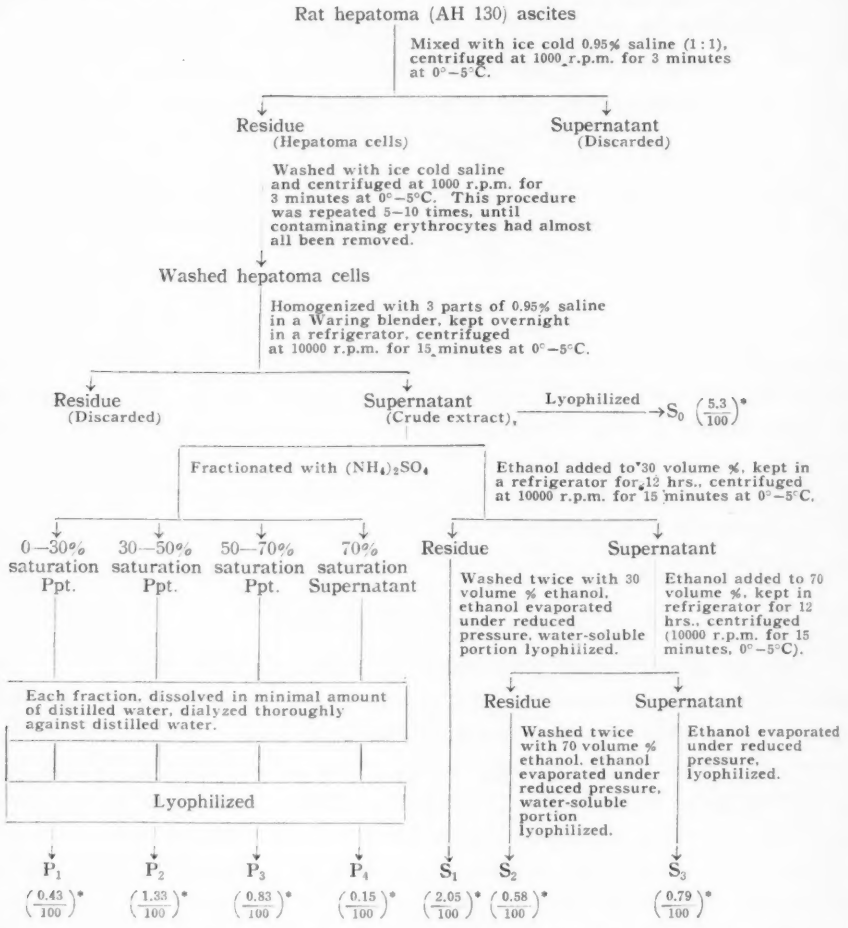
Each fraction was dissolved in distilled water and filtered by a membrane filter before use to eliminate bacterial contamination. The nitrogen content of the solution to be tested was determined by the micro-Kjeldahl method (modified by Parnas).

Results

1. *Comparison of growth-promoting effect of different fractions of ascites hepatoma*

* A part of the research expenses was paid by the Scientific Research Fund of the Ministry of Education of Japan.

Table 1. Preparation of "Oncotrephin" from rat ascites hepatoma (AH 130).



* Numbers in parentheses indicate yield of fraction designated in dry weight per wet weight washed cancer cells.

cells prepared by ethanol precipitation. Lyophilized crude extract of tumor cells, fraction S_0, showed scarcely recognizable promoting effect on strain L cell growth in tissue culture, even at a concentration of 0.054 mg (nitrogen) per ml of culture medium (Fig. 1). Fraction S_1 prepared from fraction S_0 had a rather inhibitory effect on the propagation of strain L cells at a concentration of 0.02 and 0.004 mg (nitrogen) per ml (Fig. 2), and fraction S_3 also showed a similar inhibitory effect at a concentration of 0.01 and 0.002 mg (nitrogen) per ml (Fig. 3). Fraction S_2, however, considerably accelerated the propagation of strain L cells at a concentra-

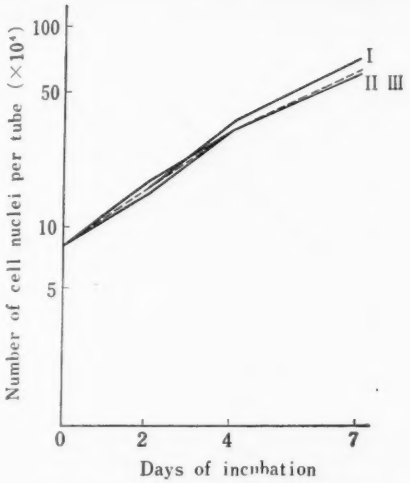


Fig. 1. Effect of fraction S_0 prepared from rat ascites hepatoma (AH 130) on proliferation of strain L cells.

I : 0.054 mg/ml* II : 0.01 mg/ml*
III : 0.002 mg/ml* ---: Control
* Final concentration of test material added to the culture medium, designated as mg N.

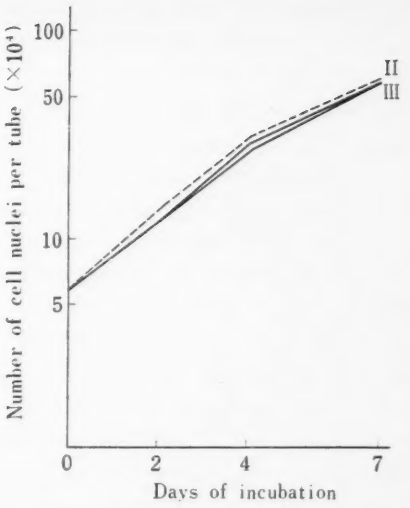


Fig. 2. Effect of fraction S_1 prepared from rat ascites hepatoma (AH 130) on proliferation of strain L cells.

II : 0.02 mg/ml* III : 0.004 mg/ml*
---: Control
* Final concentration of test material added to the culture medium, designated as mg N.

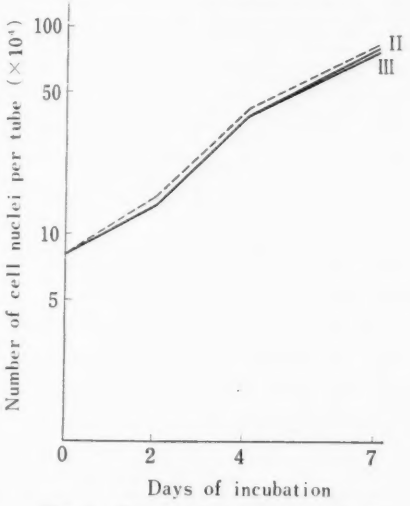


Fig. 3. Effect of fraction S_3 prepared from a rat ascites hepatoma (AH 130) on proliferation of strain L cells.

II : 0.01 mg/ml* III : 0.002 mg/ml*
---: Control
* Final concentration of test material added to the culture medium, designated as mg N.

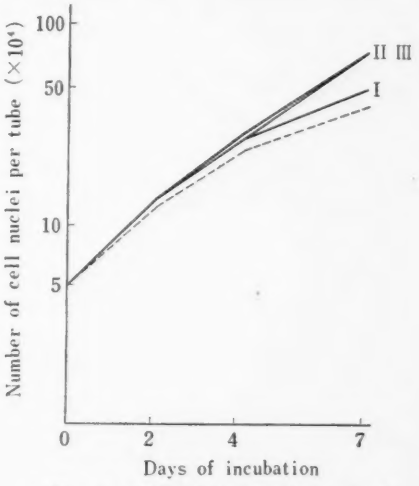


Fig. 4. Effect of fraction S_2 prepared from rat ascites hepatoma (AH 130) on proliferation of strain L cells.

I : 0.054 mg/ml* II : 0.01 mg/ml*
III : 0.002 mg/ml* ---: Control
* Final concentration of test material added to the culture medium, designated as mg N.

tion of 0.01 and 0.002 mg (nitrogen) per ml (Fig. 4). It caused an increase of 80% in the number of cells over the control. The growth promoting effect was reduced when fraction S_2 was added at a higher concentration (0.054 mg nitrogen per ml).

2. ***Influence of heat upon the effect of fraction S_2.*** An aqueous solution of fraction S_2 was heated in boiling water bath for 30 minutes, and the growth-promoting effect on strain L cells was compared with that of non-heated material. The same effect was observed in both cases (Fig. 5).

3. ***Comparison of growth promoting effect of different fractions of ascites hepatoma cells prepared by $(NH_4)_2SO_4$ precipitation.*** Fraction P_1 showed rather inhibitory effect and fractions P_2 and P_4 showed scarcely recognizable promoting effects on the propa-

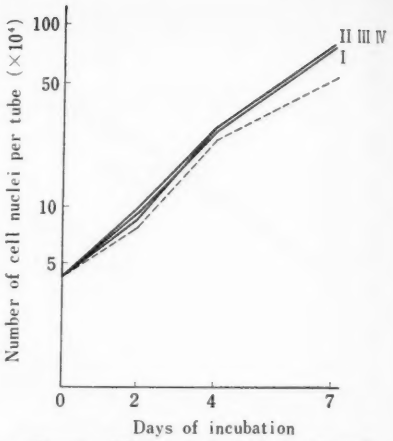


Fig. 5. Effect of heated fraction S_2 prepared from rat ascites hepatoma (AH 130) on proliferation of strain L cells.
I : fraction S_2 (0.01 mg/ml*) II : fraction S_2 (0.002 mg/ml*) III : heated fraction S_2 (30 min. in boiling water bath, 0.01 mg/ml*)
IV : heated fraction S_2 (0.002 mg/ml*)
--- : Control
* Final concentration of test material added to the culture medium, designated as mg N.

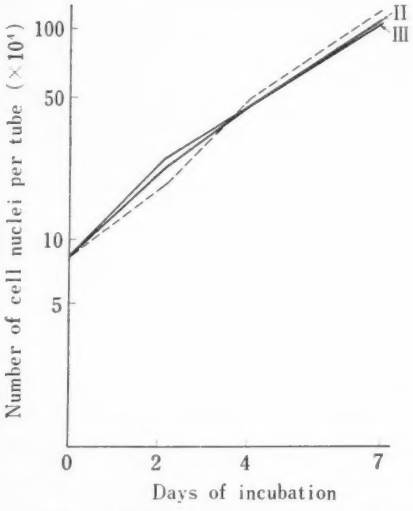


Fig. 6. Effect of fraction P_1 prepared from rat ascites hepatoma (AH 130) on proliferation of strain L cells.
II : 0.004 mg/ml* III : 0.0008 mg/ml*
--- : Control
* Final concentration of test material added to the culture medium, designated as mg N.

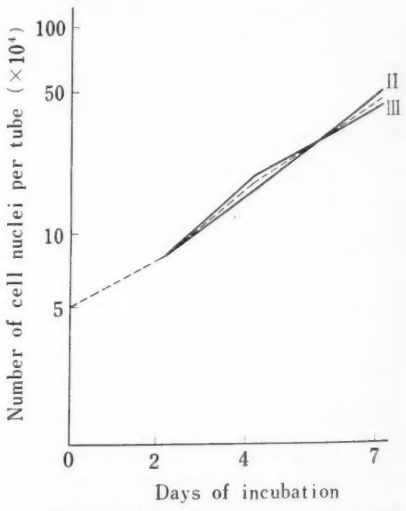


Fig. 7. Effect of fraction P_2 prepared from rat ascites hepatoma (AH 130) on proliferation of strain L cells.
II : 0.004 mg/ml* III : 0.0008 mg/ml*
--- : Control
* Final concentration of test material added to the culture medium, designated as mg N.

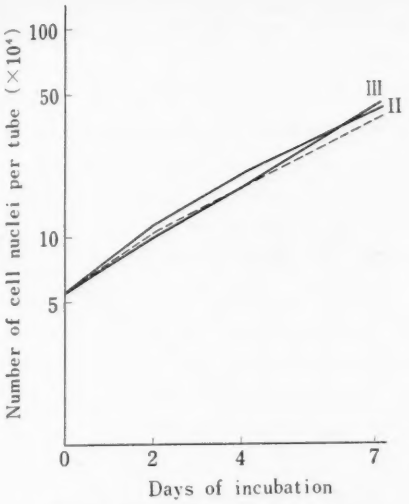


Fig. 8. Effect of fraction P_4 prepared from rat ascites hepatoma (AH 130) on proliferation of strain L cells.
II : 0.002 mg/ml* III : 0.0004 mg/ml*
--- : Control
* Final concentration of test material added to the culture medium, designated as mg N.

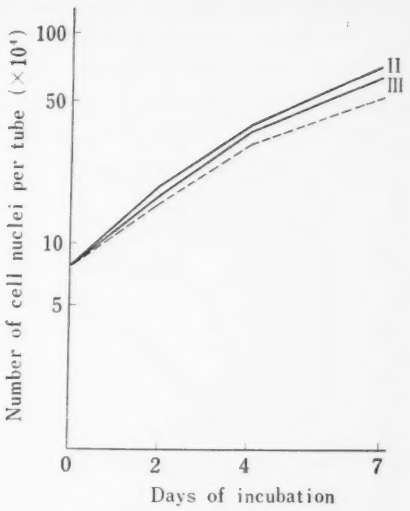


Fig. 9. Effect of fraction P_3 prepared from rat ascites hepatoma (AH 130) on proliferation of strain L cells.
II : 0.004 mg/ml* III : 0.0008 mg/ml*
--- : Control
* Final concentration of test material added to the culture medium, designated as mg N.

gation of strain L cells in tissue culture, as shown in Figs. 6, 7 and 8. When fraction P_3, precipitated by 50~70% $(NH_4)_2SO_4$, was added at a concentration of 0.004 mg (nitrogen) per ml, an increase of ca. 30% in the cell count over the control value was observed (Fig. 9).

4. ***Growth-promoting effect of fraction S_2–P_3.*** Fraction S_2 was further fractionated with $(NH_4)_2SO_4$ into four fractions (S_2–P_1, S_2–P_2, S_2–P_3, S_2–P_4). The yields of fractions S_2–P_1, S_2–P_2 and S_2–P_4 were too low to allow assay. The yield of fraction S_2–P_3 was sufficiently rich to assay. At a concentration of 0.02 mg (nitrogen) per ml, fraction S_2–P_3 produced an increase by 25% in the number of cells over the control (Fig. 10).

5. ***Effect of dialysate and residue of fraction S_2.*** Ten ml of solution of fraction S_2

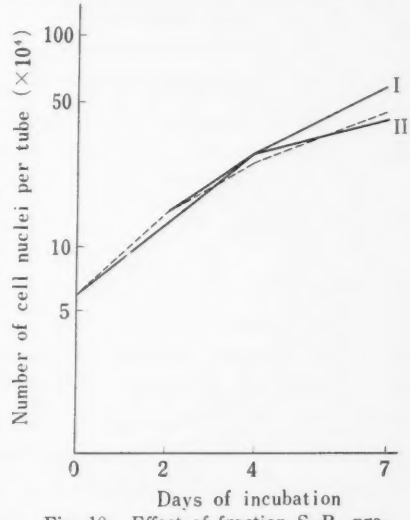
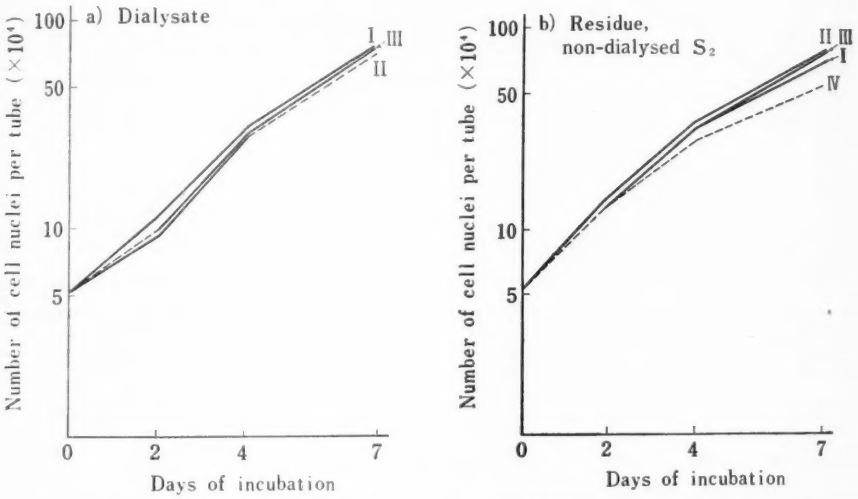


Fig. 10. Effect of fraction S_2-P_3 prepared from rat ascites hepatoma (AH 130) on proliferation of strain L cells.
I : 0.02 mg/ml* II : 0.004 mg/ml*
--- : Control
* Final concentration of test material added to the culture medium, designated as mg N.

(0.6 mg nitrogen per ml) was dialyzed for 48 hours against a hundred parts of distilled water. The whole dialysate and the residue were lyophilized respectively. The each lyophilized material was dissolved in 10 ml distilled water, and the activity of each was compared. While the dialysate caused almost no increase in cell number of strain L cells, the residue increased the cell number over the control by 35% (Fig. 11 a) and b)).



I : Undiluted solution of lyophilized dialysate of fraction S_2 added in 10% concentration to the culture medium.
II : ··· in 2% concentration ···
III : ··· in 0.4% concentration ···
--- : Control

I : Undiluted solution of non-dialyzed fraction S_2 (0.5 mg nitrogen/ml) added in 2% concentration to the culture medium.
II : ··· in 0.4% concentration ···
III : Undiluted solution of lyophilized residue of fraction S_2 (0.4 mg nitrogen/ml) added in 2% concentration to the culture medium.
IV : ··· in 0.4% concentration ···
--- : Control

Fig. 11. Comparison of effects of dialysate, residue and non-dialyzed material of fraction S_2 prepared from rat ascites hepatoma (AH 130) on proliferation of strain L cells.

6. *Effect of fraction S_2 prepared from normal rat liver.* Instead of washed rat ascites hepatoma cells, perfused liver excised from normal adult hybrid rats was treated according to the procedures described in Table 1. The effect of the fraction precipitated by 30~70% ethanol from crude extract of the normal rat liver, corresponding to the S_2 fraction described above, was tested. No effect was observed at a concentration of 0.002 mg (nitrogen) per ml. At higher concentrations, cell

sheets sloughed off so that no propagation could be observed (Fig. 12).

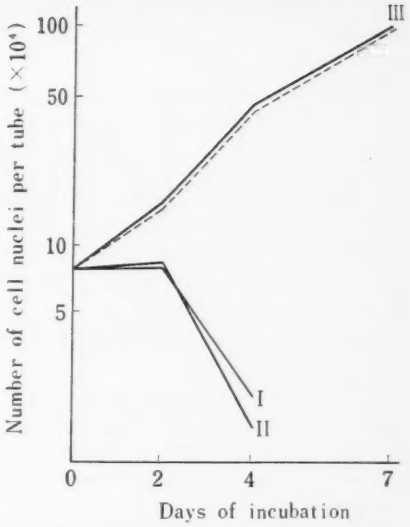


Fig. 12. Effect of fraction S_2 prepared from normal rat liver on proliferation of strain L cells.
I : 0.05 mg/ml* II : 0.01 mg/ml*
III : 0.002 mg/ml* --- : Control
* Final concentration of test material added to the culture medium, designated as mg N.

Discussion

Much research has been done to isolate growth-promoting substances from various tissues including tumor and embryonic tissues, utilizing the tissue culture technique (see Paschkis' review[8]). For example, Hoffman and his collaborators[1),2)] investigated the growth-promoting effect of various embryonic and adult tissue extracts upon a tissue culture of chicken fibroblast. They found that the saline extracts of various adult tissues of some animals were highly active, whereas the similar material from other species was ineffective. They also found that the growth-promoting effect of tumor extract upon chicken fibroblast cultures was no greater than that of extracts of the corresponding normal tissues. Moreover, extracts from a rat or hamster tumor inhibited the growth of chicken fibroblast in tissue culture. Werner[9)] attributed these results to the coexistence of an inhibitory factor in the extracts of tumor tissues. According to him, if the inhibiting factor is removed by fat solvents the growth-promoting effect can be demonstrated.

As above-mentioned oncotrephin is a substance (or substances) originally isolated from certain rapidly growing malignant tumors of man. It was not only characterized by its mitosis promoting activity on mouse epidermal cells *in vitro*, but also by its effect in promoting growth of strain L cells. In the present study oncotrephin isolated from experimental rat hepatoma was added to the culture medium and its effect upon strain L cells was reconfirmed. We also demonstrated that the analogous fraction from normal liver homogenate precipitated with 30~70% ethanol does not promote the growth of strain L cells.

Summary

Oncotrephin, isolated from a rat ascites hepatoma (AH 130), was added to the culture medium, and its growth-promoting effect, at optimal concentration, for strain L cells was reconfirmed. Neither heating for 30 minutes in boiling water

bath nor dialysis abolishes the growth-promoting effect of oncotrephin upon strain L cells.

The authors wish to thank Miss E. Yosioka sincerely for her helpful collaboration.

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